The Short Term Effects of Vasoactive Agents and Calcium Regulating Hormones on Bone Blood Flow and Mineral Exchange in the Rat

Evelyn Gray BSc. Hons.

PhD.
University of Edinburgh
1990

STATEMENT

This work is an account of original experiments designed and executed entirely by the author . Except where clear acknowledgement is made to the work of others, the results presented in this thesis have been obtained solely by the candidate.

E. Gray

Erratum

1. For calcemic / calcemia please read calcaemic / calcaemia
2. In all graphs and figures for begining read beginning

CONTENTS

ABSTRACT

It is now believed that there is a significant association between skeletal blood flow and the movement of other ions in and out of bone. Bone is capable of regulation of this flow but this is affected by factors such as physiological stimuli, arterial blood pressure and humoral agents which can override this ability. The effects of vasoactive substances, noradrenaline and ATP and calcium regulating hormones, PGE2, PTH and calcitonin on bone blood flow and strontium clearance have been investigated in an experimental model in the rat.

In order to proceed with this work smaller studies were undertaken to develop and validate the technique used to measure flow and clearance. These included;

1. validation of choice of cannulation site,
2. investigation of rat bone haematocrit,
3. determination of an appropriate withdrawal rate.

Cannulation of the carotid artery and insertion of the cannula into the ventricle provides adequate mixing of the microspheres, withdrawal at the caudal artery (0.197ml/min) an adequate reference sample. The haematocrit work suggests that a correction factor may be required when using this procedure as the haematocrit of rat bone is consistently less than that of the caudal artery. However the results in this study are too variable to incorporate this finding in the bone blood flow and clearance work.

The main study involved the use of radioactive microspheres to estimate bone blood flow and strontium-85 for clearance in rats weighing approximately 350 grammes. This involved cannulation of the carotid and caudal arteries with injection of the agent and microspheres via the carotid and reference blood samples withdrawn from the caudal.

For each animal blood flow and strontium clearance in bone, arterial blood pressure and blood flow in muscle were measured and from these strontium extraction and vascular resistance in bone and muscle were calculated.

Infusion of noradrenaline resulted in a significant decrease in blood flow and an increase in blood pressure, while ATP caused a significant decrease in only blood pressure. Neither agent had any effect on strontium clearance. Parathyroid hormone produced a significant decrease in both bone blood flow and blood pressure with a general trend of decreasing clearance with increasing concentration (not significant). While administration of PGE2 significantly reduced blood flow, pressure and strontium clearance. Calcitonin had no effect on any variable apart from strontium clearance. This was dependent upon the degree of change at the 50U. dose which represented an increase of 30% while at the other doses clearance remained relatively constant.

With all groups, there was a significant relationship between blood flow and strontium clearance, but the slope of regression was significantly different from the control group for PGE2 only. This shows that PGE2 was having a direct effect on bone independent of its effect on blood flow. In the normal untreated animal a change in flow is reflected by a change in clearance, indicating that some vascular mechanism is involved. The PTH, ATP, calcitonin and noradrenaline animals all demonstrate this same pattern suggesting that these also act through some vascular mechanism, either directly or indirectly. But PGE2 has a direct effect on strontium clearance indicating that some other mechanism may be involved, possibly through a non-vascular effect. This would therefore invalidate the use of clearance measurements as estimates of bone blood flow.

ACKNOWLEDGEMENT

The author would like to take this opportunity to express her gratitude to the many people who have assisted throughout all stages of preparation of this thesis.

I would like to thank Professor S.P.F. Hughes for extending the facilities of the Department of Orthopaedic Surgery and for the opportunity to carry out this research.

I would also like to acknowledge the advice and guidance provided by Dr I.D. McCarthy, and admire his tact in curbing my wider flights of fancy.

To the various members the technical staff : Mrs B.Wyatt, Mr A.Sher and Mr B. Fleming I acknowledge the technical assistance.

The author would like to express her sincere gratitude to :

Mr R.K. Strachan

Mrs C. Darlington

Dr H.T. Law

Mr A.Gibson

for their help, critisicm and friendly discussion throughout the project.

Additionally I would like to express my thanks to my husband, Owen, who has been not only a constant source of encouragement and understanding, but in addition has greatly lightened the burden of compiling this thesis. He has willingly given up many evenings to provide asistance of the highest possible standard without complaint and with very little reward.

I would also like to express my gratitude to the Medical Research Council for providing the funding for this research and the financial assistance provided by Edinburgh University in the form of a Gordon Lennie Bursary.

III Illustrations

a) FIGURES

b) TABLES Page

LIST OF ABBREVIATIONS

a.a. -- amino acid

ATP -- Adenosine Tri-phosphate.

B1 -- blood pressure at the point of cobalt microsphere injection.

B2 -- " " " " beginning of the experimental run.

B3 -- " " " " point of tin microsphere injection.

B4 -- " " " " point of maximum drug effect

Cal. -- Calcitonin.

CPM -- Counts per microsphere.

DPM -- Disintegrations per minute.

D.F. -- Degree of freedom.

ECG -- Electrocardiograph

F -- Flow.

ml/min/g.-- millilitre per minute per gramme.

Nor. -- Noradrenaline.

PGE2 -- Prostaglandin E2

PTH -- Parathyroid hormone (bPTH = bovine parathyroid hormone).

SD -- Standard deviation.

S.G.B.-- Specific Gravity of Blood (1.05).

Tech -- Technique

Var -- Variance.

V.R. -- Vascular resistance.

INTRODUCTION

1.1. Bone function and cellular composition

Bone is a complex arrangement of cells matrix and mineral which functions as an accessible mineral store and structural support. Its structural role is obvious: the skeleton serves as a framework upon which the soft tissue of the body are supported so that the form of the body and an erect posture can be maintained. The storage of minerals in bone is a secondary function and to some extent is incompatible with its structural role.

The control of mineral metabolism in the body is dependent on processes occurring in the kidneys, intestine and bone. Each playing an important role in maintaining the mineral balance, particularly plasma calcium levels. Within bone, formation and resorption occur continuously and play an important part in the maintenance of this balance. Hormones causing resorption bring about the dissolution of both the mineral and organic phases, affecting the skeletal content and its ability to support the soft tissues correctly. In fully developed bone there is a balance between bone resorption and bone formation, while in growing bone an inbalance of these causes remodelling of the bone, but growth in length is the result of more complex processes (Smith 1984). Defects in any of these processes or metabolic disorders can result in abnormal bone which does not function efficiently with respect to its role of structural support and mineral storage. Of the three major components of bone it is the cells which mediate these conflicting demands. Each cell type is primarily associated with a specific function and together these are responsible for the formation, mineralisation and remodelling of bone. The osteoblast's main function is to lay down the osteoid matrix and to some extent play a part in the mechanism of calcification. An essential role in resorption is played by the osteoclast, although the mechanism of this action is still today under review. There is no question however, of the osteoclast's essential part in the process, since so many

factors, e.g. parathyroid hormone, that affect resorption produce marked changes in this cell type. Finally with regard to the osteocyte views concerning its function are controversial. However it, is agreed that this cell is necessary for the maintenance of bone as a living tissue. The activity of each cell is influenced by hormones, local agents and mechanical factors.

1.2. Vascular supply

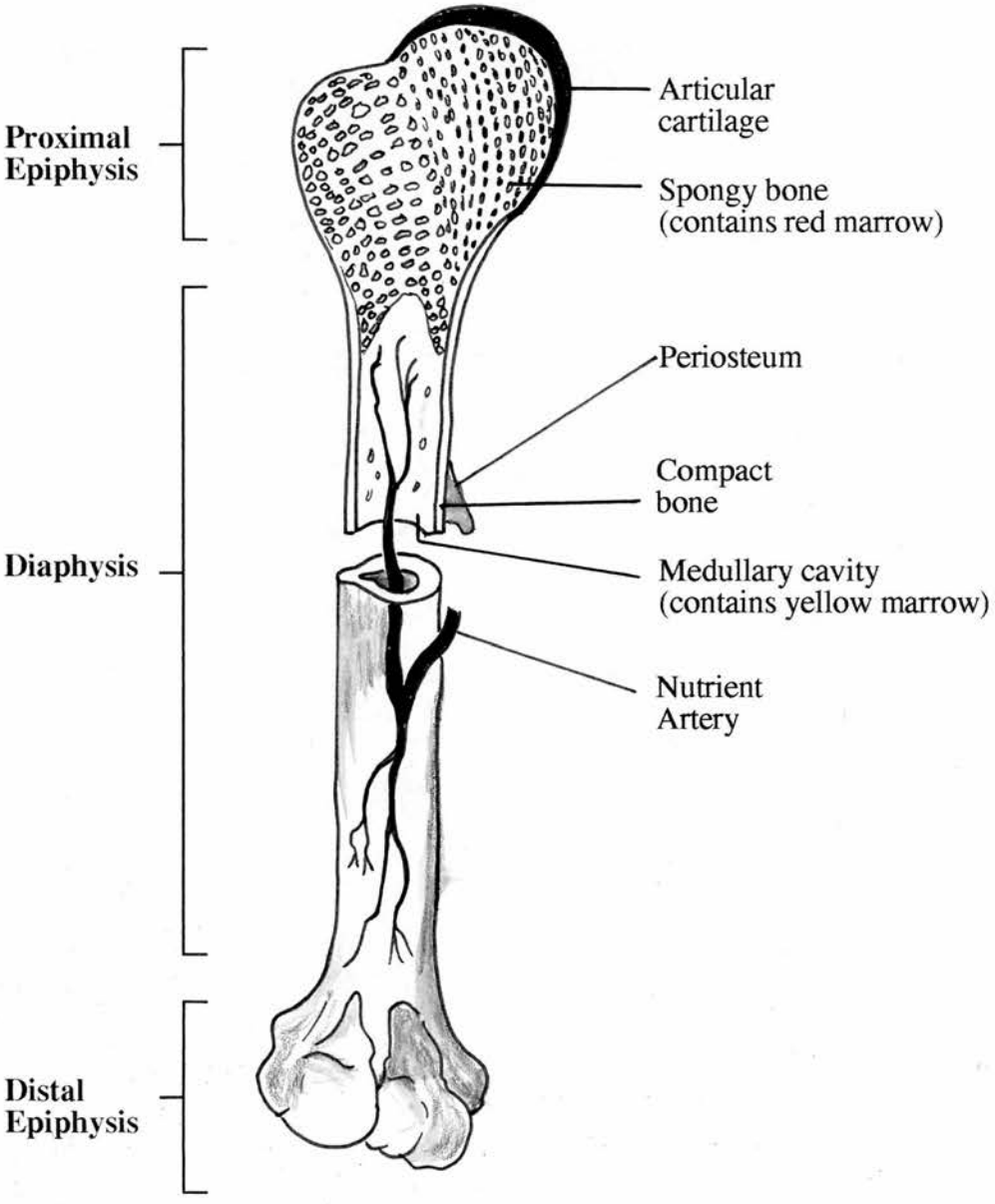
Throughout life a two-way chemical traffic between blood stream, cells and matrix is required to maintain this cellular activity. Thus the vascular supply of bone is important with respect to this exchange.

The literature cites the long bone as the general model for description of the blood supply to bone. This represents the chief type of osseous tissue and is a fundamental component of the appendicular skeleton. A long bone consists of three regions: the metaphysis at either end made up of mostly cancellous bone surrounded by a protective layer of compact bone and the diaphysis, a shaft of compact bone which contains the medullary cavity (Figure 1.1.). The blood supply consists of three basic components: (a) the nutrient artery, (b) the metaphyseal combined, after closure of the growth plates, with the epiphyseal arteries, and (c) the periosteal arteries. Brookes considers the principle nutrient artery and the metaphyseal arteries to represent the main components of the bone nutrient system (1971).

Nutrient artery

The nutrient artery and its branches supply at least two thirds of the cortex by a centrifugal method, from the endosteal to the periosteal surface. This principle of the nutrient artery system always holds even though there are major anatomical variations within the mammalian species (Rhinelander 1972). Arising directly from the systemic circulation, the nutrient artery enters the diaphysis by a distinct foramen.

Figure 1.1. **Osseous tissue**
Schematic diagram of mammalian long bone showing macroscopic appearance



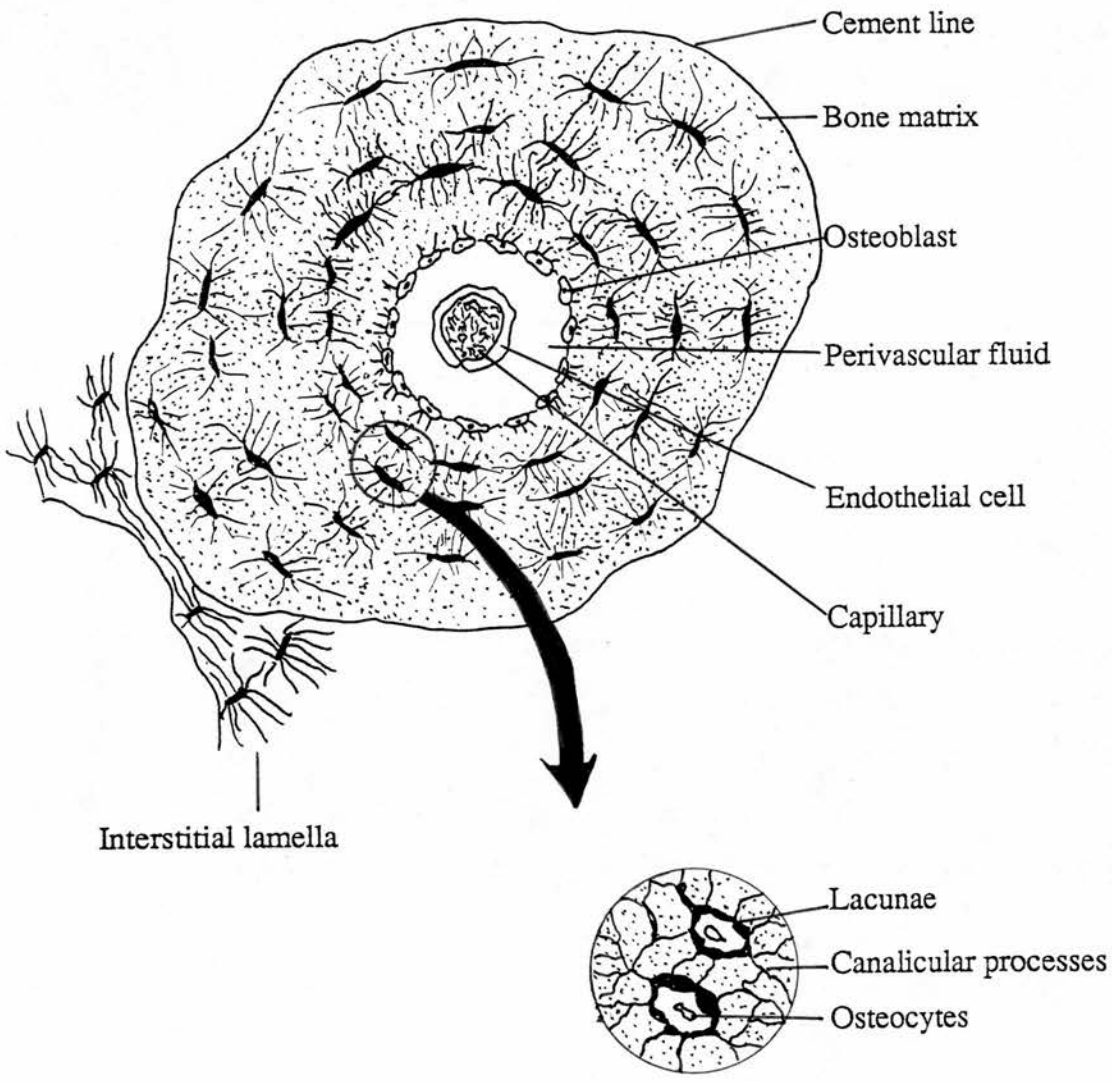
Adapted from Tortora and Anagnotsakos
Principles of Anatomy and Physiology

In the medullary cavity it branches into ascending and descending branches (medullary arteries) subdividing into radially orientated arterioles. These penetrate the endosteal surface of the diaphysis where they go into Haversian canals.

Haversian systems tend to develop as animals age i.e. in the young stages bone is mainly composed of lamellar bone which develops into Haversian systems, the number of these systems varying between species (Figure 1.2.). This is to some extent a compromise since Haversian bone is less efficient in terms of surface area of vascular channels per cm^3 (Halstead 1974). Additionally the osteocytes can become cut off from the nutrient source when the cement lines of the new canal is laid down. However the one main advantage of the Haversian system over the lamellar structure is its ability to be remodelled. In adult bone a balance is struck between the efficiency and strength of the lamellar bone with the adaptability of Haversian bone and thus adult cortical bone contains both lamellar and Haversian systems. The number of Haversian systems varies from species to species and is dependent to some extent on how bone is laid down. In man and dogs there are a large number of these systems while in rodents this number decreases and the percentage of lamellar bone increases.

Each Haversian canal is contained within an osteon, which is considered to be the basic morphological unit of compact bone. The degree of mineralisation, size of Haversian canal and activity of cells in the canal differs between osteons. Unless bone is being resorbed (combined action of many cells including the osteoclast) or formed (osteoblast) the Haversian canals are lined with 'resting osteoblasts'. The centre of each Haversian canal contains one, and occasionally two blood vessels which ultrastructurally resemble capillaries (Cooper et al 1966). These authors noted that where two vessels were seen one was often smaller and thicker than the other. This confirmed the observations of Branemark (1959) who remarked that when two vessels were present, the flow of blood was in opposite directions. An adequate blood supply is maintained by transverse canals which connect each osteon with the major endosteal and periosteal vasculature.

Figure 1.2. Diagram of the cortex of long bone



A haversian system of cortical bone, between each system there are interstitial lamella.

Metaphyseal arteries

The metaphyseal system, as well as supplying the metaphysis, acts as an important adjunct to the medullary circulation. Within the medullary cavity the metaphyseal arteries anastomose with the terminal branches of the ascending and descending medullary arteries, thus providing the medullary cavity with a metaphyseal supply.

Efferent vascular system

Drainage for this extensive capillary network is provided by the components of the efferent vascular system. This includes (a) in the diaphyseal region the emissary veins and the vena comitans of the principle nutrient artery, (b) in the epiphyseal region the veins accompanying the epiphyseal artery, (c) and marrow sinusoids which drain directly into the central venous sinusoids.

1.3. Regulation of blood flow in bone

In the mid-19th century sympathetic nerves were observed in the blood vessels supplying bone by M. Gros, this finding to be confirmed by Ottolenghi in the early 1900's (Tøndevold 1983). Their activity appears to arise through changes in arterial blood pressure and denervation or stimulation of carotid and aortic baroreceptors. In the seventies these were demonstrated to affect blood flow, in particular reducing the flow upon stimulation (Shim et al 1972). Simultaneously measuring systemic blood pressure, blood flow and intermedullary pressure of bone, these authors found a good correlation between blood flow and medullary cavity pressure, thereby supporting Shaw's earlier view that a positive correlation existed (Shaw 1963). This evidence implies that intramedullary pressure is bone blood flow dependent and reflects the changes in the haemodynamics of bone.

Tøndevold (1983) suggested that medullary pressure therefore reflects the circulatory

conditions in bone and that measurement of this was a good indicator of bone blood flow. Measuring both arterial and intra-osseous pressure in tubular bones in dogs he identified the ''threshold'' point at which medullary pressure dropped in response to arterial pressure. The medullary pressure was constant at all measured sites if the arterial pressure was kept above 80mmHg. Five to ten mmHg below this critical level there was a sharp fall in the medullary pressure.

Since medullary pressure reflects changes in blood flow the above suggests that bones are capable of maintaining constant flow rates if the arterial pressure is 80mmHg. Upon stimulation the sympathetic nerves present in bone blood vessels can affect this flow. Thus the factors responsible for this action are important when considering bone blood flow.

1.3.1. Physiological stimuli

Brookes (1971) suggested that venous efflux through the nutrient veins of bone would be promoted by muscular activity. Using microspheres as indicators of bone blood flow Gross et al (1979) investigated this, finding that in conscious dogs exercising caused an increase in bone vascular resistance as a result of bone vessel constriction, in contrast to profound vasodilation in the skeletal muscle. This indicates that bone participates in the redistribution of blood flow during exercise.

A more recent study detected a delayed hyperaemia after submaximal exercise, with the duration of the exercise possibly important in determining the extent and the time of blood flow increase (Tøndevold 1983). During exercise a steal phenomenon may come into action, vasodilation of the muscle decreasing the effective inflow to the medullary region of bone. This delayed hyperaemia could be a repayment response to compensate the bone for some built up metabolic deficits.

While investigating the relationship between bone blood flow and the clearance of labelled substance, Schoutens (1979) found that flow could be modified over a wide range of physiological values by heating or cooling the limbs. Mean blood flow

increasing by 88% in the warmed tibiae and decreasing by 66% in the cooled tibiae compared with the value at rest. During the physiological stresses of exercise bones appear to participate in the redistribution of blood. Since exercise results in increased muscle temperature one would expect the heating of a limb to result in similar vasodilation.

1.3.2. Arterial blood gas composition

Like other tissues bone requires oxygen for the normal metabolic processes. Therefore the oxygen content of blood and its ability to take up the by-product of these processes is important when considering blood flow in bone.

Decreasing arterial oxygen tension in the blood supplying dog hind limbs results in vasodilation causing an increase in blood flow of the femoral artery (Ross et al 1962). This means that a vasodilation in the tissues of the hind limbs occurs, increasing the general blood flow, with the presence or absence of oxygen acting as a stimulus. Two mechanisms could be responsible for this: firstly the cells of the tissues could compete for the available oxygen limiting the supply to the smooth muscle cells of blood vessels so they are unable to maintain constriction. Or secondly, the actual lack of oxygen could cause vasodilatory substances to be released. Tondevold (1983) demonstrated that the relationship between intraosseous pressure and arterial oxygen tension begins to come into action at an oxygen tension of 75mmHg. Above this point intraosseous pressure is independent of the arterial oxygen tension. A reduction in bone perfusion occurs which could be due either to an increased sympathetic tone and/or to steal phenomenon from other tissues, a shunting of blood from supplying bone to other more vital organs arising when the oxygen tension falls. The vasodilation of muscle and surrounding tissue increasing flow and directing blood from the bone vascular network could give rise to this phenomenon.

Bone, in particular bone marrow, consumes oxygen and produces carbon dioxide as a by-product. The process of osteogenesis is dependent on carbon dioxide (CO_2) tension

and is increased by raising this over a minimum (Wilmer 1965). Erickson et al (1979) have identified a linear relationship between arterial blood gas tension and medullary blood gas tension, in particular for carbon dioxide. This means that the carbon dioxide content of bone seems to be correlated to the arterial content. However there appears to be no active regulation of this.

1.3.3. Hormonal

Research into humoral regulation on blood flow has been extensive. These studies suggest that as in other vascular beds hormonal production influences bone circulation. The vasoconstrictive action of adrenaline was demonstrated by Drinker and Drinker (1916). Using a method of bone marrow perfusion, vasomoter nerves were stimulated with adrenaline. Extending this study Tran et al (1978) examined the effect of drug treatment on the intramedullary pressure in dog tibiae. Adrenaline was found to cause an increase in perfusion pressure and a fall in medullary pressure, consistent with its action as a vasoconstrictor. The administration of alpha-inhibitors reduced or suppressed this increase indicating the possibility of intraosseous vasoconstrictor alpha-receptors.

Noradrenaline has been found to have a direct constrictor effect on bone vessels, the magnitude of this constrictor response being dependent on the initial flow rates (Gross et al 1979, Driessens and Vanhoutte 1979). The walls of blood vessels serving bone contain vascular smooth muscle cells which contract in the presence of alpha-adrenergic agonists. Since the action of both adrenaline and noradrenaline is abolished by alpha-adrenoytic drugs it would appear that these are acting on the smooth muscle cells causing this constriction. McCarthy et al (1985) investigated bone blood flow before and after infusion of noradrenaline or adenosine tri-phosphate (ATP). As expected noradrenaline decreased bone blood flow and increased bone vascular resistance by acting as a vasoconstrictor, whereas ATP increased bone blood flow and decreased bone vascular resistance, implying that ATP dilates resistance vessels. This vasodilatatory effect of adenosine on marrow vessels had previously been noted by Gross et al in 1979.

It would appear that the action of noradrenaline, adrenaline and ATP is probably a direct effect of the respective agent on vascular smooth muscle surrounding the arterioles, mediated through adrenergic receptors present in smooth muscle cells.

Evidence is accumulating that parathyroid hormone (PTH) influences vascular resistance as well as the rate of bone resorption. In an attempt to separate this hypotensive property from the hypercalcemic action of PTH Pang et al have conducted a series of studies. They have identified the possible amino acids involved in each process, the synthetic fragments of bPTH containing amino acids 24-34 and 24-28 having hypotensive activity in rats and dogs. Finding that blood flow was increased in renal cortex, heart, liver and stomach, they concluded that PTH has a direct vasodilatatory action on vascular smooth muscle (Pang et al 1981,82,84). If this is the case one would expect bone blood vessels to react in the same manner, thereby increasing blood flow to bone. However Driessens et al (1981) found that PTH had no direct effect on canine blood vessels and only increased flow during the hypercalcemic phase of action. Since PTH was not found to alter significantly the smooth muscle cell response to adrenergic activation, its action would not appear to be through this mechanism, unlike calcitonin and hydrocortisone, whose effects on bone is due to some extent on their direct effect on bone blood vessel smooth muscle cells.

It is therefore apparent that bone is highly responsive to both external and internal stimuli. These give rise in some cases to vasodilation or vasoconstriction, possibly through action on the smooth muscle cells of bone blood vessels. The result is control of blood flow, pressure and to some extent the systemic blood volume. Apart from affecting the ability of bone to regulate blood flow, some of these factors, in particular the humoral, can influence the exchange of minerals between blood and bone. Here the question is can blood flow alone alter this exchange ?

1.4. Mineral Exchange

Before discussing the control of this process it is important to note that ''exchange'' when used to describe the movement of ions has different definitions depending upon the authors citing the phrase. This can be broken down into two descriptions, firstly exchange is ''any process involving equal and opposite rates of transfer of calcium ions to and from one microscopic volume of bone'' (a microscopic volume = 10μm cubed) (Marshal et al 1959, Rowland 1966). Secondly exchange is used to describe the movement of ions in relation to hydroxyapatite crystals i.e a precise mechanism is concerned (Groer and Marshal 1973). Here I refer to the later definition, the process of calcium movement in relation to the bone surface.

As stated in section 1.1. bone has two main functions, mineral storage and structural support. With reference to these mineral exchange can be discussed under the headings skeletal and mineral homeostasis. The factors controlling both are complex and to some extent interrelated.

Skeletal homeostasis may be considered as the maintenance of skeletal integrity through growth and remodelling of the skeleton. This is affected by hormones, mechanical stress, pressure and other external factors.

On the other hand mineral homeostasis is the control of the level of minerals throughout the body. It is maintained by a complex system of controlling factors acting on the skeleton, absorption process of the gut and excretion by the kidney. The mechanisms for the exchange of minerals and the factors affecting this in bone are presented here.

1.4.1. Bone-blood barrier ?

Does a membrane exist separating bone fluid from extracellular fluid ? In 1971 Neuman argued that the circulating fluids could not be in equilibrium with bone surfaces; thus a membrane must exist separating a perivascular space from the bone fluid. Talmage (1969) suggested that a layer of cells separates the Haversian capillaries and the

mineralised matrix at the periphery of the canal. This results in two fluid compartments, one of which is directly associated with mineralised tissue while the other is part of the body's extracellular fluid reservoir.

Originally the primary characteristics of the bone fluid compartment were considered to be the low calcium and the high potassium levels in the blood compared to the extracellular fluid. A calcium pump could account for the low level, the mineral actively transported against a concentration gradient out of the cell, while the excess potassium has been suggested to be under cellular control (Geisler 1969, Ramp 1971, Scarpace 1976 a).

Recent work by Pinto and Kelly (1984) argues against this excess potassium, suggesting that it can all be accounted for by cell volume. The implication is that potassium exchange is a cell dependent process as other tissues. Therefore arguing that there is not a barrier controlling the potassium flux. These authors conclude that the there is no barrier separating the extracellular fluid of bone and like other tissues, that bone has a vascular space, an interstitial fluid space and an intracellular fluid space. Thus the only barrier separating the plasma and the interstitial space is the capillary wall. This however disagree with the findings of Scarpace (1976 b), who investigated the influx and efflux of calcium and phosphorous across the membrane into calvarial bone. He found that these fluxes were concentration-dependent, a model of passive diffusion. This supports the concept of a barrier controlling the fluxes of certain minerals. However the exact relationship of the osteocytes (the cell type separating two compartments), the extracellular fluid and the adjacent bone matrix is not known. The proximity of these suggest the possibility of a close physiological relationship, perhaps that of mineral homeostasis.

1.4.2. Pathway for exchange

For minerals and ions to pass to the bone matrix they first have to pass through the capillaries to the interstitial fluid. It is generally accepted that minerals and ions

exchange by diffusion and water by filtration or osmosis. If it is accepted that there is some form of control of transport from capillaries to bone then the structure of this barrier is important when considering the passage of minerals.

Essentially a capillary is a tube like structure with walls composed of a single layer of endothelial cells. There are three types: continuous, fenestrated and discontinuous. The thin-walled nature of the vessels allows lipid soluble substances to pass across the the capillary by diffusion processes. Numerous authors have investigated this transcapillary exchange, demonstrating that water and water soluble lipids do indeed pass through pores in the capillary wall by passive diffusion (Pappenheimer 1953, Crone 1963 and Davies et al 1976). It has been pointed out that this passive diffusion was dependent upon molecular size, the larger the molecule the larger the diffusion coefficient and thus the slower the movement across the capillary wall (Hughes et al 1977). Within the capillary the blood flow is also important in determining the equilibrium point of this diffusion. At low flow rates the concentration of solute decreases continually along the capillary length so that an equilibrium point is reached before the distal end of the capillary. Thus the transport rate is directly proportional to the flow and is said to be flow-limited (McCarthy et al 1983). On the other hand at higher flows there is less chance of this point being reached and extraction becomes diffusion-limited.

Within bone the capillary structure varies depending on the location. Thus this is important when considering exchange at the different locations i.e. marrow and cortex. The microvascular network of marrow changes with age, in the adult animal the marrow consists of adipose tissue (yellow marrow) while in the immature animal this is composed of active haemopoietic tissue (red marrow). There is variation in the capillary type between these two types of marrow the size of vessel varying according to the functional state. In the active haemopoetic tissue the vessels are small and sinusoid in nature (Trueta and Harrison 1953). These authors were not able to demonstrate open fenestrations (pores) between the endothelial cells. But Zamboni and Pease (1961) found small gaps in the sinusoid wall structure of rat long bone. These could represent

artifacts and be of no importance to exchange, however the lack of basement membrane underlying the reticular cell cytoplasm means that marrow sinusoids would present minimal hindrance to exchange.

In cortical bones the capillaries are contained within the Haversian canal (page 2 - 1.2.). Hughes and Blount (1979) have shown that in the rat the cortical capillaries are similar to those in skeletal muscle, although a basement membrane surrounding these was not identified. Cooper et al (1966) noticed that intercellular capillary clefts were present in cortical capillaries, spaces of 175 A between adjacent endothelial cells could be measured. Thus the transcapillary movement of substances could occur through two pathways: 1. passive diffusion of lipophilic substances through these clefts and 2.transport by pinocytic process across the endothelial cells for larger molecules such as albumin which cannot diffuse through transcapillary clefts.

Crone (1963) used a method of multi tracer injection into the vascular system as a means of obtaining a ratio of permeabilities for substances e.g. inulin to sucrose in muscle. When this technique was applied to bone the relationship between permeability and diffusion coefficients for lipo-insoluble substances (diphosponate, sucrose, sodium, fluorine and strontium) was established. In this case a linear relationship was demonstrated for the lipo-insoluble complexes while the fat-soluble substances showed much higher permeabilities. This establishes that diffusion through bone capillaries is dependent on molecular size (Hughes 1980).

Large molecules such as horse radish perioxidase have been observed to penetrate the canalicular lacunae region of bone after injection intravenously (Doty and Scofield 1972). This supports the findings of Cooper et al (1966) and implies that the transport of larger molecules is by pinocytic processes.

Thus the normal mechanism of solute uptake in cortical bone appears to be by passive diffusion through the capillaries of the haversian system. These solutes pass through capillary clefts to the interstitial fluid and pass on to bone. In the marrow the presence of clefts has not been proved however the thin-walled nature of the vessels provides minimal hindrance to passage.

1.4.3. Biological factors affecting mineral exchange

Bone is a storage site in the body for calcium, phosphate, sodium, carbonate and other ions. This function, as a reservoir, assists in the maintenance of the concentration of these ions in the tissue fluids. All are of importance in the metabolic processes of the body but, with respect to its bone it is the mechanism of calcium homeostasis that has been widely reviewed. The ways in which calcium is deposited on the organic matrix, and the mechanisms that control the calcium equilibrium in the body being of particular influence. Calcium moves in both directions between plasma and bone surfaces, the quantity exceeding that involved in bone turnover. Thus the extracellular fluid is constantly replaced. In order to maintain constant plasma levels there must be some mechanism that keeps these fluxes in equilibrium. One concept involves two linked mechanisms, firstly hormonal control of skeletal turnover and secondly extracellular regulation i.e. hormonal action on the gut and kidney, the linking of the systems ensuring that the action of one does not negate the action of the other (Parfitt 1976). Here it is the hormonal mechanism of control that is discussed.

Hormonal action

It has been demonstrated in vitro that administration of PTH stimulates bone resorption. The evidence from work with numerous experimental systems supports the theory that PTH increases the recruitment of osteoclasts from the osteoprogenitor cells, leading to the activation of new remodelling cycles and thus to an increase in bone turnover. In the rat, using scanning electron microscopy Jones et al (1970) found the resorbing surface of bone to increase after PTH treatment but that the nature of the surface remained the same. The early action of PTH appears to be the activation of cyclic AMP, noradrenaline inhibits this while propanolol has no effect (Heershe et al 1974).

Apart from this increased resorption PTH administration affects plasma calcium levels. Webster et al (1974) found that in culture, calcium and phosphate ions are released in a 2:1 ratio with PTH treatment. The release of calcium is linear and occurs over such

a wide range of concentrations that this can be used for an assay in vitro. Grubb et al (1977) demonstrated that in vivo plasma calcium levels are raised with PTH treatment. The work of Parsons et al suggests that this rise occurs after an initial period of transient decline. Movement of calcium from the plasma to bone occurring within two minutes of injection decreasing the plasma calcium levels. Dacke and Shaw (1987) disagree with this initial influx of calcium into bone finding that PTH inhibits the exit of acutely injected 45-Ca from the plasma, thereby supporting the elevation of plasma calcium through this inhibitory mechanism. The timing of this response is dependent on the dose and varies in sensitivity between species. Dacke and Shaw (1987) found that 45-Ca uptake was inhibited within three minutes in chicks with prostaglandin E2 (PGE2) and PTH treatment, while in rats the timing of the response to PTH was similar but less sensitive. Unfortunately this study did not allow bone blood flow to be measured directly.

The timing of these two events i.e. histological changes caused by bone resorption and the hypercalcemic response have been widely investigated along with the third characteristic action of PTH, that of a hypotensive response. It appears that these are distinct actions of the hormone and these occur at different intervals after administration. Raisz and Niemann (1969) found that changes in plasma calcium could be seen within hours but that at least 24 hours were required before histological evidence of increased resorption in response to PTH could be seen. This was confirmed in the work of Tatevossian (1973) who noted that the hypercalcemic response was rapid and occurred long before any morphological changes.

With respect to the hypercalcemic action Pang et al (1982, 83) studied this, along with the hypotensive effect, identifying the portions of the hormone involved in each. The bPTH 1-34 portion was required for each response but the 24-28 a.a. portion was crucial for the hypotensive action and the 1-2 a.a. for the hypercalcemic action. These authors also found that the hypotensive response was immediate, whereas the hypercalcemic response required time to develop.

In some respects the effects of prostaglandins are similar to those of PTH, both appear to act through cAMP and calcitonin inhibits both. The demonstration by Klein and Raisz (1970) that prostaglandins were able to resorb bone directly in vitro has been confirmed by several groups. In normal and diseased states of bone prostaglandins are thought to have important effects on mineral release . However unlike PTH reports on the effects of PGE2 on plasma calcium levels are conflicting. Several authors have noted that PGE2 administration to rats and dogs failed to produce a hypercalcemic response (Beliel et al 1973, Klein et al 1970) while other authors have found that in vivo PGE results in a similar if generally smaller hypercalcemic responses as PTH (Vanderweil et al 1979, Voelkel et al 1975). The work of Dacke and Shaw (1987) supports the hypercalcemic action of the hormone. They suggest that a decrease in clearance of 45-Ca label in response to PGE2 and PTH could result from inhibition of calcium uptake by bone, this inhibition representing a mechanism underlying the hypercalcemic action .

It is clear that prostaglandins like PTH are potent bone resorbers but that prostaglandins' role in regulation of calcium release in bone is uncertain. It is possible that the action of prostaglandins is through a secondary mechanism where the the action of other agents requires the synthesis of prostaglandin (Martin 1983).

Vitamin D is also important in the control of plasma calcium. Production of this vitamin appears to be controlled by secretion of PTH, which then acts on the kidney. Vitamin D3 then moves to the gut and stimulates calcium transport across the intestinal epithelium. This in effect increases plasma calcium concentration the level acting as a controlling factor in PTH secretion (Williams 1981) It has also been demonstrated that this vitamin effects the skeleton, a deficiency affecting calcification (Vaughan 1981). Inhibition of bone resorption results in a decline in the release of minerals.

Calcitonin, the most potent inhibitor, lowers both calcium and phosphate levels in the plasma (Tashjian 1965). The greatest effect occurs between 30 minutes and 3 hours. When ingested the effect on bone cells is immediate (Vaughan 1981). This hypocalcemic response is more marked in young animals and is greatly reduced in older animals.

This action however is not just the opposite of PTH but a complex interaction with the hormone in order to control plasma concentration.Other work shows that calcitonin probably also plays a vital part in the protection of the skeleton from excessive osteoclastic resorption (Stevenson et al 1979)

1.5. Theses objectives.

In summary, bone is capable of regulation of blood flow but this is affected by factors such as physiological stimuli, arterial blood pressure and humoral agents which can override this ability. Many investigators have shown that vaso-active and calcium regulating hormones affect flow in bone. However little work has been done to identify the relationship between drug concentration and response. This study was set up to identify this relationship. Recent work has shown that PTH and PGE2 have a rapid inhibitory effect on calcium uptake in bone. Both these substances are known to have vascular effects. Therefore this study also investigated these vascular effects with respect to altered uptake of minerals to see if a change in blood flow alone could alter this uptake by bone.

These factors have direct orthopaedic applications. The technique of skeletal scanning relies on labelling compounds eg 18-F and Tc-99m that are incorporated into the bony matrix of hydroxyapatite. Skeletal uptake of these bone seeking radionuclides is flow dependent (McCarthy et al 1980). If the flow can alter the uptake of minerals then its suggests that the uptake of the labelled compound will be affected. Similarly if the metabolic activity of bone itself can affect this extraction process then the use of clearance measurements as a method of estimating bone blood flow in vivo is questionable.

Rats were chosen as the experimental animals for three reasons.

1. Previous bone blood flow and mineral clearance studies have used this species and therefore direct comparisons can be made.

2. The rat is a mammalian species and therefore more comparable with human bone then would be if a non mammalian species was used. Although rat bone is not comparable in terms of structure i.e. a high percentage of lamellar bone compared to human, the general blood supply to the bones is similar.

3. The expense and access to large numbers was also a factor in the choice of the experimental animals. A more comparable bone structure would be provided using a dog model but this would have involved a high expenditure and have severely limited the number of animals in each group.

The work fell into three categories.

1. Vascular supply and Histological presentation

The structure and vascular supply to long bone has been well documented. This varies to some degree from species to species so here the intention was to establish the vascular supply and examine the Haversian / lamellar content for rat bone. Also to view the bone types which form the basic unit of long bone i.e.the trabecular and cortical bone. Thereby enabling a comparison to be made with the findings of others authors in these areas.

2. Development and validation of the technique to measure blood flow and strontium clearance in the rat

This section reviews the problems in establishing the correct protocol for the measurement of bone blood flow and mineral clearance in the rat. The results of an initial control group are presented and the associated problems discussed. These include choice of cannulation site, pump setting, bone haematocrit, method of drug administration and the error parameters associated with the technique. Each topic is tackled in turn and small

studies were set up in an attempt to validate each choice. The findings of these are documented in the conclusion and are discussed with reference to the experimental protocol.

3. Measurement of blood flow and mineral clearance in a rat

This represents the main point of the work to establish the effects of calcium regulating hormones and vaso-active agents on :

a. Blood flow,

b. Vascular resistance,

c. Mineral exchange,

d. Blood pressure.

The intention was to attempt to quantify effects of these agents creating dose response curves for each. In addition to examine if a change in blood flow can alter the uptake of mineral by bone or if mineral exchange can be altered independently of blood flow. The plasma calcium levels were examined to study whether effects on mineral exchange in bone could alter these values.

VASCULAR SUPPLY AND HISTOLOGY

2.1. Introduction

It has been established that bone is provided with an adequate supply of blood by the nutrient artery, metaphyseal arteries and the epiphyseal arteries. The principle of a centrifugal method of blood movement from the endosteal to the periosteal surface has been described by Brookes and Harrison (1957). Methods of investigating the layout of blood vessels in bone include indian ink perfusion (Irving 1965) and injection of radio-opaque media into the main vessels followed by x-rays (Rhinelander 1968).

With reference to rat bone the illustration of this vascular network has been well reviewed by Brookes (1971). This author describes the nutrient arteries as perforating the cortex of the diaphysis and the bone extremities. These then pass into the bone marrow and divide to form the medullary arteries, while the compact bone of the diaphysis is supplied by cortical blood vessels.

The use of barium sulphate (radio-opaque) allows easy visualisation of the nutrient vessel and its ramifications in long bone. Immediately after removal and cleaning, the bones can be x-rayed and the vascular network viewed, although decalcification of the bone can make the vessels appear clearer. The micropaque however is too viscous to fill any but the larger vessels. The extensive processing that is required to visualise the vascular network using ink includes clearing of the bone, a time consuming and tedious technique which limits its use.

Histological preparations allow further detailed examination of the vascular supply and nature of rat bone . The foundation of all good histological preparations is adequate and complete fixation. This is required to prevent putrefaction and autolysis of the samples, without damaging the tissue in any obvious manner. Thus the tissue should be as close to the living state as possible. With respect to bone the calcium salts present in the matrix form a barrier to sectioning .

Once fixed, bone can easily be cut into thick sections (100μm) either transversely or longitudinally, then x-rayed or stained to examine the bone type i.e. compact or cancellous bone. Thinner undecalcified sections can be cut however this is more difficult, but decalcification of bone allows easier examination of the cellular nature. This involves the dissolving of the calcium salts by treatment with acid leaving behind the organic matter and cellular components in order to section the tissue. The criteria of a good decalcifying agent are :

(i) The complete removal of calcium salts.

(ii) The absence of any damage to tissue cells or fibres.

(iii) Non-impairment of subsequent staining processes.

Once cleared the bones are then embeded, sectioned and stained. The above criteria have been taken into consideration with respect to the processing of rat long bone. EDTA was used to remove the calcium without damaging the tissue. This allowed basic staining techniques to then be used ie. Toluidine blue.

In order to visualise the capillary network, blood supply, lamellar / Haversian strucures and the cellular make up of the rat bone barium sulphate perfusion has been used along with sectioning of decalcified and calcified bone.

2.2. Barium sulphate perfusion

2.2.1. Methods

A saturated solution of barium sulphate was made by dissolving the powder (Barium Sulphate Precipitated, Fisons Scientific equipment) in neutral buffer .

After anaesthesia (sodium pentabarbitone 60mg/ml - 0.1ml per 100g body weight, injection into the abdominal cavity) the rat was prepared for perfusion. The abdominal cavity was opened allowing access to the thorax, this was then cut along its length and the diaphragm retracted displaying the heart. A needle was then inserted into the left atrium and through this the barium sulphate solution was injected under pressure (20ml

per 400g animal). Immediately before this the animal was given an overdose of Sagatal. The vena cava was cut during this procedure to prevent a build up of pressure and to allow drainage of the circulatory system. Both femora and tibiae were removed and placed in 10% neutral buffered formalin to be fixed.

After an initial x-ray, to determine the distribution of the barium sulphate (40KV for 4 minutes using Kodak Technical Pan Film 4415 and HC1100 developer. Figures 2.1. and 2.2.) the bones were decalcified using EDTA (saturated aqueous solution of ethylene-diamine tetra-acetate 10 per cent). The whole bone required approximately a week but the end point was determined chemically. The determination of the end point was important to ensure that the bone was not exposed to the decalcifying agents longer than necessary. Testing for calcium ions dissolved in the decalcifying fluid adequately indicates the end-point.

1. 5ml of decalcifying fluid nearly neutralised with Sodium Hydroxide bubbled with Nitrogen
2. Add 1ml of 5% sodium or ammonium oxalate

Turbidity of the fluid indicates calcium is present, absence of turbidity after five minutes indicates that the fluid is free of calcium.

Once neutralised and washed the decalcified bones were then re-X-rayed (35KV for 4 minutes Figures 2.1. and 2.2.)

2.2.2. Results

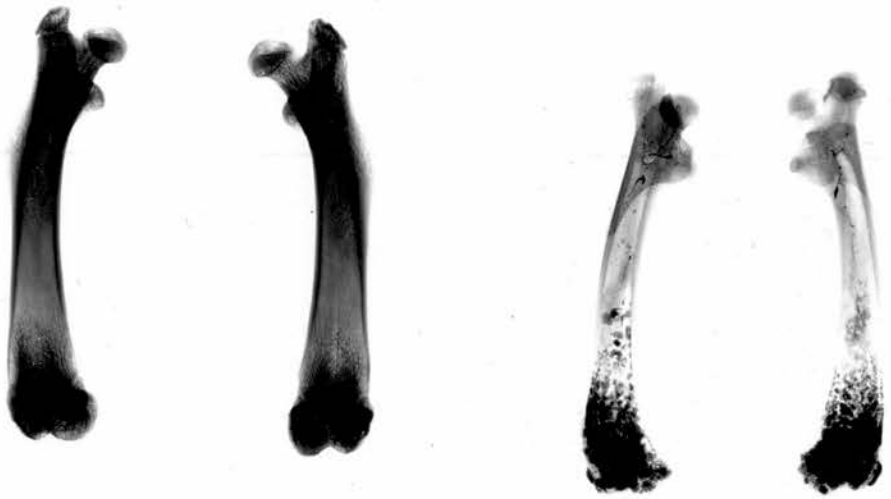
The x-rays of calcified and decalcified tibia and femur are shown in figures 2.1 and 2.2. The principle nutrient artery with its branches ascending and descending with in the medulla can be seen in these x-rays (Figs 2.1 and 2.2.). The path of some of the main medullary branches can be followed to the epiphysis.

Unfortunately in figure 2. there is an area of clouding in the femur head. This is likely to have been caused by the bursting of the finer blood vessels under the pressure of the fluid and thus the barium sulphate has escaped the vessels.

Figure 2.1. X-ray of calcified and decalcified rat tibiae showing main arteries (scale 2x)

Calcified **Decalcified**

Figure 2.2. X-ray of calcified and decalcified rat femora showing nutrient artery and medullary arteries (scale 2x)



Calcified

Decalcified

2.3. Histology of normal rat bone

Prior to sacrifice an untreated animal was given a bolus of tetracycline (2ml). The rat was then sacrificed and the femora and tibiae removed.

These were then processed using the following methods.

1. Fixation ---- The bones were placed in 10% neutral buffered formalin.

Calcified sections were then cut before further processing as this removed the tetracycline. The remaining bones were then processed as follows.

2. Decalcification ---- The bones were treated with EDTA and tested to ensure complete decalcification using sodium oxalate.

3. Dehydration ---- The bones were then taken through alcohol to absolute alcohol to remove the aqueous fixative and any tissue water. This involved processing in 70% alcohol, 90% alcohol and finally several changes of absolute alcohol.

4. Clearing ---- The bones were then placed in histoclear to remove the dehydrating agent .

5. Embedding ---- Finally the samples were embedded in paraffin wax to allow sectioning. The bones were then cut in cross sections (100µm) and stained

6. Staining ---- Toluidine Blue a general purpose stain used to highlight the cellular content and structure of the bone.

2.3.1. Results

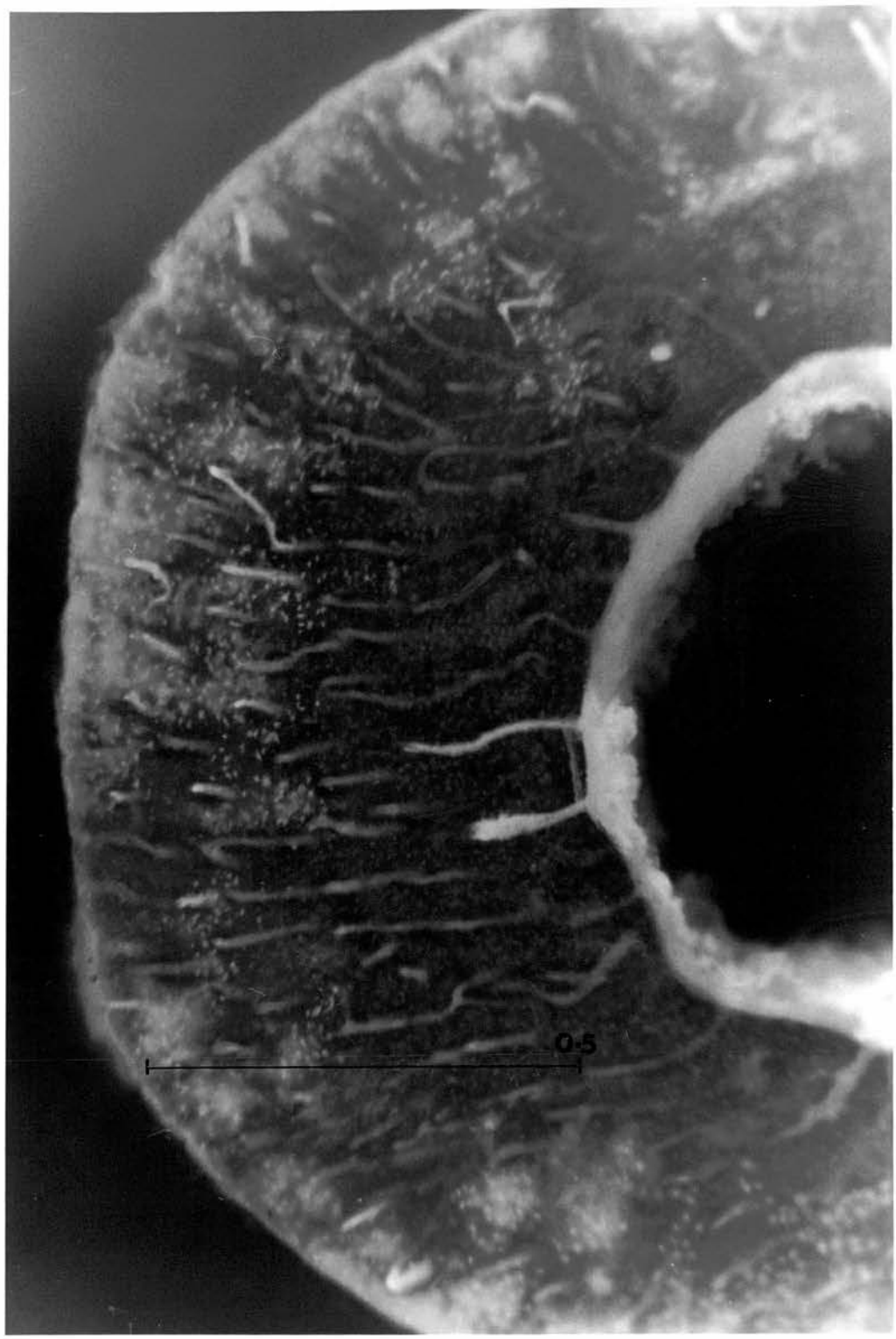
Figure 2.3 is a cross section of rat tibia showing the general structure of cortical bone. The particular type of bone in the tibiae can be seen in the following figure (Fig 2.4). Both Haversian and lamellar bone are present in the rat tibia, these occur in approximately a 1:1 ratio. Of particular interest in figures 2.3 and 2.4 is the high proportion of circumferential lamella on the endosteal surface compared to that of the interstitial lamella. The section in figure 2.5. shows cells and vascular channels (which are

probably similar to Volkmans canals). These radial endosteal canals cross the lamellar bone towards the sites of secondary ossification (Haversian bone). This means that the bone has a better blood supply than would be expected following secondary osteogenisis. This process involves the creation of new cement lines which normally restricts the Haversian blood supply. These medial canals provide a second blood supply thereby contributing to the total that the bone receives. Thus even the middle portion of the bone is fairly vascular.

Figure 2.3. Cross section of rat tibia (100μm) stained with toluidine blue (magnification X40)

Figure 2.4. Polarised unstained cross section of rat tibia (100μm) (magnification X 40)

Figure 2.5. Tetracycline fluorescent labelled section of rat tibia (magnification X 30)



2.4. Discussion

The x-rays of the tibia and femur are similar if less informative than those of Brookes (1971). Thus these appear to substantiate the facts mentioned in the introduction. That is that the nutrient artery enters the bone then divides into ascending and descending branches which supply the medulla. These vessels then enter the cortex, supplying at least two thirds of this area. The vessels which supply the cortex are to small to fill with the media and therefore this is not actually demonstrated by the figures. Similarly the x-rays do not provide information on the centrifugal method of blood supply, however the blood vessels can be seen to originate within the medullary canal and divide outwards to towards the endosteal surface.

The photographs of the tibial sections highlight the lamellar nature of rat bone. Although this type of bone exists in approximately the same proportion as the Haversian type, this is a higher proportion than is found in human bone. The presence of radial endosteal canals is important since its shows that rat bone is fairly vascular in nature. However the number of these are less than that seen in human bone.

DEVELOPMENT AND VALIDATION OF THE TECHNIQUE TO MEASURE BLOOD FLOW AND STRONTIUM CLEARANCE IN THE RAT

3.1. Introduction

The measurement of bone blood flow is complicated by the nature of the circulation of bone, the number of capillaries and veins forming a complex network that creates a multitude of difficulties. Although there are numerous methods for estimating this variable most involve the use of radioactive labels and fall into one of three categories. Each of these have certain advantages and disadvantages which are discussed here.

a. Clearance of "bone seeking" tracers as an estimate of bone blood flow

Clearance can be defined as the removal of the tracer from blood during its passage through the organ of interest. Assuming that in bone the extraction of the tracer is complete or at least occurs at a constant rate, a fixed proportion thus accumulates in the bone. It has been claimed that 18-Fluorine is completely extracted in a single circulation (Van Dyke 1967, Wootton 1978, Humphrey et al 1982). The validity of Wootton's technique has been questioned with particular reference to the maximum transit time through bone and the minimum recirculation time (Tothill and Hooper 1984). For the single passage measurement technique to be valid there must be sufficient time for any of the tracer that is not taken up to leave the bone (transit time) yet recirculation must not be allowed to occur. Tothill shows that the maximum transit time through bone is greater than the minimum systemic recirculation time. Therefore invalidating the use of this technique to measure extraction ratios in rabbits.

The extraction ratio of unity for 18-Fluorine has been questioned by Brookes (1979) who states that 'this has never been demonstrated in the case of any bone-seeking isotope'. Copp and Shim (1965) support this view, at least for strontium-85. These authors measured the extraction of this isotope with a multiple dilution technique,

finding that in a five minute period the extraction ratio was 0.76 and not unity. Yet in 1963 Shim assumes an extraction ratio close to unity when estimating the blood flow in the rabbit humerus and the tibiofibula.

It has in fact been demonstrated that the extraction of diffusible tracers are flow rate dependent (Crone 1963, Renkin 1959, Tothill et al 1984). At low flow rates this extraction is said to be flow-limited while at higher rates the extraction becomes diffusion limited since the transit time is too short for the ion to diffuse sufficiently, the equilibrium point is not reached.

Therefore the variable levels of clearance means that bone blood flow cannot be quantitatively measured using this technique. However the non-invasive nature of bone seeking radionuclides make this the best available method of estimating blood flow in human studies.

b. Freely diffusible tracers

Although this method has become well established for measurement of blood flow of specific organs it presents numerous problems in measuring bone blood flow. This technique is based on the principle that blood flow determines the removal of the introduced tracer and therefore the characteristics of the tracer are important. These include; 1. that the tracer be freely soluble thus dissolving in the blood and, 2. that the tracer flow freely having no haemodynamic effects. The method of introduction of the tracer, timing of re-circulation and heterogenous nature of bone blood flow all contribute to the difficulties of using this method to measure bone blood flow.

Lahtinen et al (1981) used 133-Xenon to estimate the washout curves of bone and marrow. However certain assumptions are included in the paper about the assignment of the fast and slow portions of the curve without sufficient validation, for example that the nonhaematopoietic compartment determines the whole washout curve in the cases where bone contains no marrow. This brings into question the method of calculation and use of the correction factors in the Lahtinen paper thus suggesting the findings presented are misinterpreted. Additionally xenon was introduced intravenously leading to label-

ling of numerous other tissues as well as bone which is not advantageous.

The one advantage of this technique is that the tracers do not have to be radioactive. A hydrogen washout technique has been used to measure blood flow in cardiac muscle, skeletal muscle and brain. Using this method Whiteside et al (1977) have demonstrated that epiphyseal and trabecular bone have approximately two fold higher rates than that of diaphyseal cortex. These regional differences in blood flow have also been demonstrated by Shim et al (1971) using a bone seeking radioisotope, and by Brookes (1967) using labelled red blood cells.

This procedure is however invasive, the bone is drilled and the electrodes placed in the holes. Therefore this method is unsuitable for use in human studies.

c. Arteriolar blockage

This method is dependent upon the blockage of arterioles by labelled tracer introduced into the arterial circulation in one passage, thus no re-circulation of the tracer should occur.

The technique involves mixing radioactive microspheres with the blood supply allowing the particles to circulate in the systemic circulation and these then trap in capillaries and arterioles in proportion to the blood flowing through the vessels. The microspheres reach their final destination in the capillary bed in one passage of blood through an organ. Arterial blood is withdrawn at a known rate before, during and for a short time after the injection of the microspheres. This gives a reference sample of mean blood radioactive concentration during the withdrawal period to compare with the activity of the tissue under investigation. After sacrificing the animals the tissue of interest is removed, weighed and then along with the reference blood sample counted for activity. From these counts the Tissue Blood Flow can be estimated,

$$\text{Tissue Blood Flow} = \frac{\text{Tissue count - Background}}{\text{Reference blood count - Background}} \times \text{Rate of withdrawal}$$

This can then be divided by the weight of the tissue to give the results in the form of ml/min/g.

Additionally if the activity of the micropheres is measured before injection the proportion of cardic output flowing to reference organs can also be calculated.

The following guide lines should be followed for the use of this technique if the estimation of blood flow is to be valid.

1. The microspheres must be well mixed and evenly suspended in the blood.

2. The microspheres must be of a size to ensure entrapment in the system during the first passage in the circulation.

3. There must be sufficient numbers of microspheres to satisfy the requirements of statistical treatments.

4. The microspheres must be of a uniform size and no preferential arterio-venous shunting should occur.

The above produces conflicting requirements. Firstly microsphere size; these have to be large enough to trap in the systemic system yet small enough to give realistic values of blood flow. Furthermore, the microspheres must not have haemodynamic effects which would alter this blood flow. However numerous studies have established the requirements which fit these criteria i.e. injection of spheres into the heart (Tøndevold 1983), optimum size of 15μm diameter (Gross et al 1979), minimum number of microspheres (393) in a sample (Buckberg et al 1971).

This technique has some advantages over (a) and (b). Firstly it allows blood flow to be estimated in the whole skeleton, individual bones and portions of bones in the same animal. Any changes in flow due to some intervention can be followed using multiple injections of different labelled microspheres. And the use of microspheres with mineral analogues allows bone clearances and blood flow to be simultaneously measured. Therefore the relationship between flow and clearance can be interpreted and drug response can be examined. It is important to note that the microspheres must not have any haemodynamic effect themselves or the use of the technique in assessing drug effect is inappropriate. This method is unsuitable however for human studies due to the radioactive nature of the microspheres and the invasive procedures involved in the technique.

3.2. General methods

3.2.1 The microspheres and isotope

This study measured both bone blood flow and mineral clearance in the same animal. Thus changes in flow as a result of treatment could be related to changes in clearance. Microspheres with different labels allows two separate measurements of bone blood flow to be made and compared. Cobalt-57 and Tin-113 (New England Nuclear) were selected because these gamma emitters have completely different energies and long half lives.

	Principal Photon Energy	Half Life
Cobalt-57	122-136 keV	271 days
Tin-113	393 keV	115 days

Thus the counts from the two sources could easily be distinguished in the gamma counter, crossover only occurring downwards i.e. tin into cobalt, simplifying the calculations. Also the long half life of these two isotopes meant that counting could be delayed when necessary, without significantly affecting the counts. This long half life ensured that the microspheres were still relatively active in the later days of dispensing when the bottles of microspheres could be months old.

Strontium-85 was chosen to estimate mineral clearance in bone. This is a calcium analogue and exchanges directly with calcium hydroxapatite crystal in the bone and therefore its clearance is representative of calcium exchange. Although calcium-45 is available this is a beta emitter and when used with gamma emitters the emissions of this isotope are difficult to distinguish. Strontium-85 is a gamma emitter the energy of which is higher than both cobalt and tin, therefore cross over occurs into both of these.

	Principal Photon Energy	Half Life
Strontium-85	514 keV	60 days

Before dispensing the microspheres the syringes were coated with silicon (Sigma). This involved drawing the silicon into each syringe and then emptying it. These syringes

were allowed to dry out before use. This was done to minimise the extent of microspehre adhesion to syringe walls. Approximately 0.2ml each of cobalt-57 and tin-113 labelled microspheres were drawn into separate siliconised syringes. This represented about 200,000 microspheres with an estimated activity of 5μCi (NEN; 0.5mCi in 20ml physiol solution). A third syringe was used to dispense 0.05ml of 85-SrCl solution, this volume has an approxi mate activity of 5μCi (Amersham 1mCi in physiol solution, made up to 10ml using saline).

Activity per microsphere

Before using a new source of microspheres the activity of individual microspheres was estimated (CPM = counts per microsphere) and then compared with the manufacturers specific activity (DPM = disintegrations per minute). This allowed the calculation of the number of microspheres to be made in each sample and acted as a secondary check ensuring that the minimum number (393) for 95% confidence limits were met.

The process involved dispensing a diluted solution of each microsphere type onto cm^2 pieces of graph paper on microscope slides. This was then covered with selotape and the slides viewed at 10x magnification in order to see the actual microspheres (Figure 3.1.). Approximately 200 microspheres were on each slide and could be easily counted by eye and three counts were made on each slide. These counts were averaged and the activity of each counted along with the standard for each isotope. The activity of each microsphere type was then estimated from the total number of spheres and the activity count.

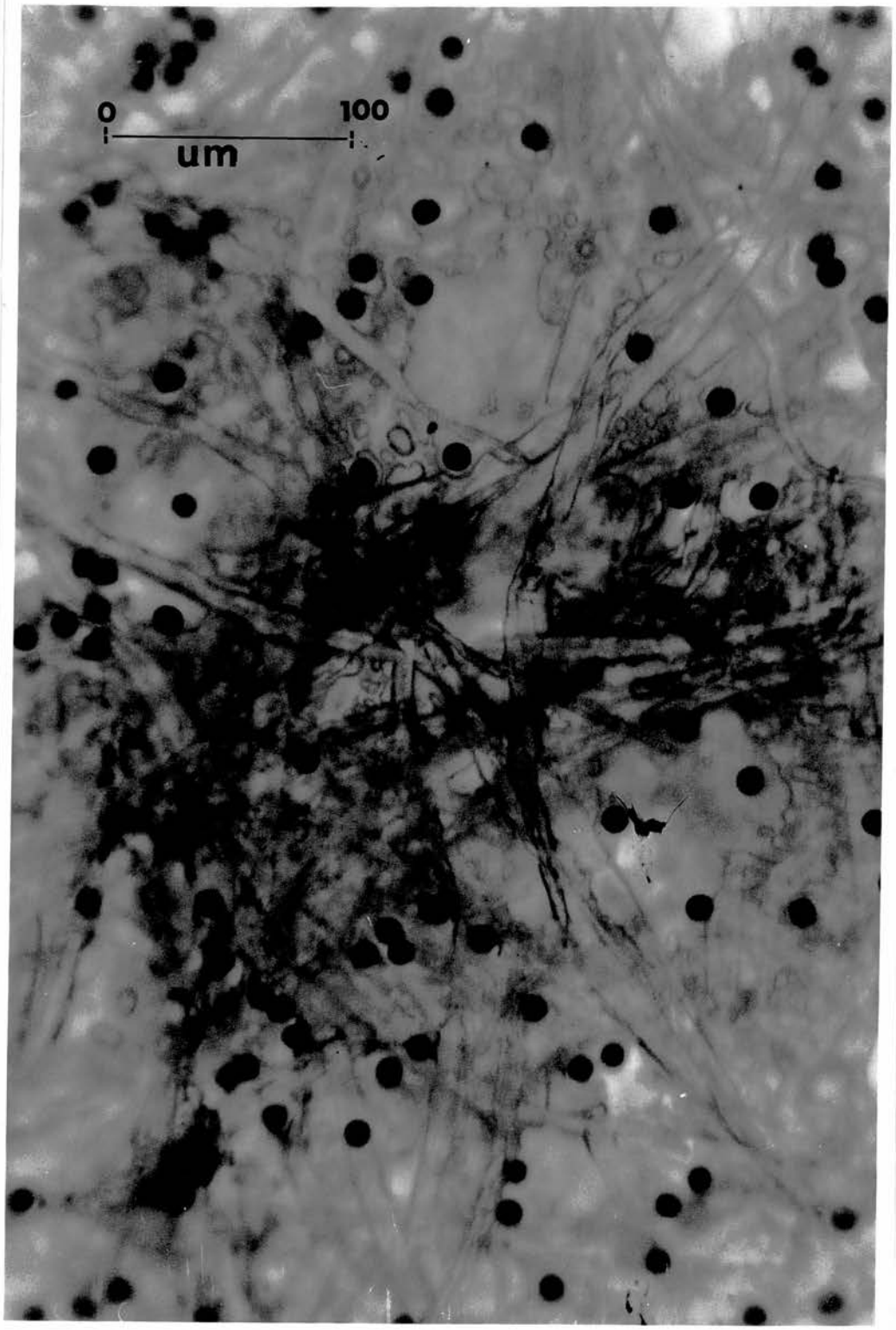
Thus for two sources the counts per microsphere from new bottles were;

			Standard
1.	Cobalt-57	66 counts per microsphere 16/11/88,	22085
2.	Tin-113	116 counts per microsphere 16/11/88.	43155

The information sheet from the manufacturer details the specific activity at their calcibration date e.g.3/11/88

Cobalt-57	3/11/88	55DPM microsphere
Tin-113		81DPM "

Figure 3.1. An example of the 15μ diameter spheres on graph paper (magnification X 100)



These two values can be compared since "count" refers to a detected "disintegration". As it is unlikely that all disintegrations would be reflected in the "count", CPM should be less than DPM. This is not the case here and could have occurred because the number of microspheres on the slides was underestimated. The microspheres were some times crushed when the Selotape was added to the slide and these could have been mistaken for artifacts and not included in the count. Additionally the presense of the Selotape occasionally made it difficult to focus, thus microspheres could have remained undetected. These figures did however allow the counts per minute to be related to the standard. In each case the counts per microsphere is over estimated, therefore the use of these values ensured that the number of microspheres in each sample was under estimated rather than overestimated. Thus a cut off level of 400 actually reflects a higher number of microspheres, ensuring that the criteria necessary for 95% confidence limits were met.

The activity per microsphere decreases with time (radioactive decay) but the standards were made from the same source and on the same date as the slides used to calculate the CPM. This means that an estimate of counts per microsphere in the standard can be adjusted each time it is counted in the gamma counter. Thus the decay can be taken into consideration and the specific activity of the microspheres can be estimated throughout the period any one bottle is used. Therefore an approximate number of counts per sample could be calculated as a check that at least 400 microspheres were present in the measured samples.

3.2.2. Dissection and cannulation

Before beginning the dissection the cannula were prepared. This involved filling the cannula with heparin (Evans 5000U in 1ml) to coat the cannula and prevent blood clots blocking the cannula. This cannula was then flushing with saline containing 0.05% heparin and connected to a syringe containing this solution.

Adult male Sprague Dawley rats weighing approximately 300-400 grammes were

anaesthetised using sodium pentabarbitone (60mg/ml) at a concentration of 0.1ml per 100g body weight. This was injected into the abdominal cavity. The response of the animal was tested by strong squeezing of its hind foot pads. When fully anaesthetised the rat was placed on an electric heating pad covered with Benchkote.

An incision was made in the top portion of the tail, only deep enough to penetrate the skin. Using forceps this was widened to expose the connective tissue, and by cutting through this the artery below was uncovered. Once the artery was identified a pair of tweezers were passed under, to allow a loop of suture (Ethicon) to be pulled through. During this procedure it was important to ensure that the vessel did not become twisted. The suture was cut in two, the lower portion of the vessel was tied off and the suture held with a pair of artery forceps. The upper tie was looped and also secured with a pair of forceps. These were either held by an assistant or latched so as to apply pressure to the artery thus cutting off the blood flow. A lateral cut was then made in the artery and a cannula (0.63mm outside diameter, Portex), was gently pushed into the artery. Once again it was essential that neither the artery or the cannula twisted. The top suture was then released and the cannula pushed until 2cm was in the artery. This was then secured using the suture to tie the vessel to the cannula. Blood was drawn up the cannula to check for adequate withdrawal and then the cannula flushed using the saline filled syringe (Figure 3.2.)

Using forceps and scissors a cut 2cm long was made in the mid line of the neck. The forceps were then used to widen this, exposing the subcutaneous tissue underneath. This fat, and associated connective tissue were teased away from this area using tweezers, thereby exposing the underlying muscle. The muscle to the left of the midline (Musculus Sternohyoideus) was gently teased away from the remaining muscle creating a 'window' access to the artery. After clearing the remaining connective tissue the artery was lifted and a suture passed underneath. This was then cut in two and the suture furthest from the heart tied, while the other was looped. The cannulation procedure was similar to that for the tail. However we attempted to insert the cannula (0.75mm outside

diameter, Portex) into the left ventricle pushing approximately 2cm into the vessel and pulling the cannula back slightly once it came into contact with the ventricle wall (Figure 3.3.).

In some of the control animals the femoral artery was also cannulated. A 2cm cut was made in the groin region of either side of the animal and opened using forceps. The connective tissue was teased away from the medial surface of the thigh and overlying muscles separated (adductor brevis and caudo-femoralis)to expose the femoral artery. The artery along with the associated nerve and vein was traced to the point where forms a Y-shaped branch incorporating the external iliac artery. A suture was passed under the vessel prior to this branching and the vessel cannulated (0.75mm outside diameter, Portex) using the method already described. Approximately 2cm of the cannula was inserted into the vessel before tieing it off (Figure 3.2.).

In each animal a control blood flow was established using 57-Cobalt microspheres, blood withdrawn from the tail artery at 0.197ml/min/g (and in some animals the femoral cannula) and thirty seconds later 200,000 microspheres injected via the carotid cannula. Blood withdrawal ceased after a total of two minutes. Next the agent was either injected or infused, withdrawal commenced, followed thirty seconds later with an injection of Tin-113 microspheres and 85-Sr. Blood withdrawal ceased after a further four minutes. At the end of the experiments the animals were sacrificed using KCl, injected via the tail cannula. Both tibiae and femora were removed quickly to minimise post-mortem migration of ions into bone (Tothill and MacPherson 1978). A muscle sample from the upper quadriceps was also removed.

Figure 3.2. Photograph showing cannulation of both the femoral and caudal arteries

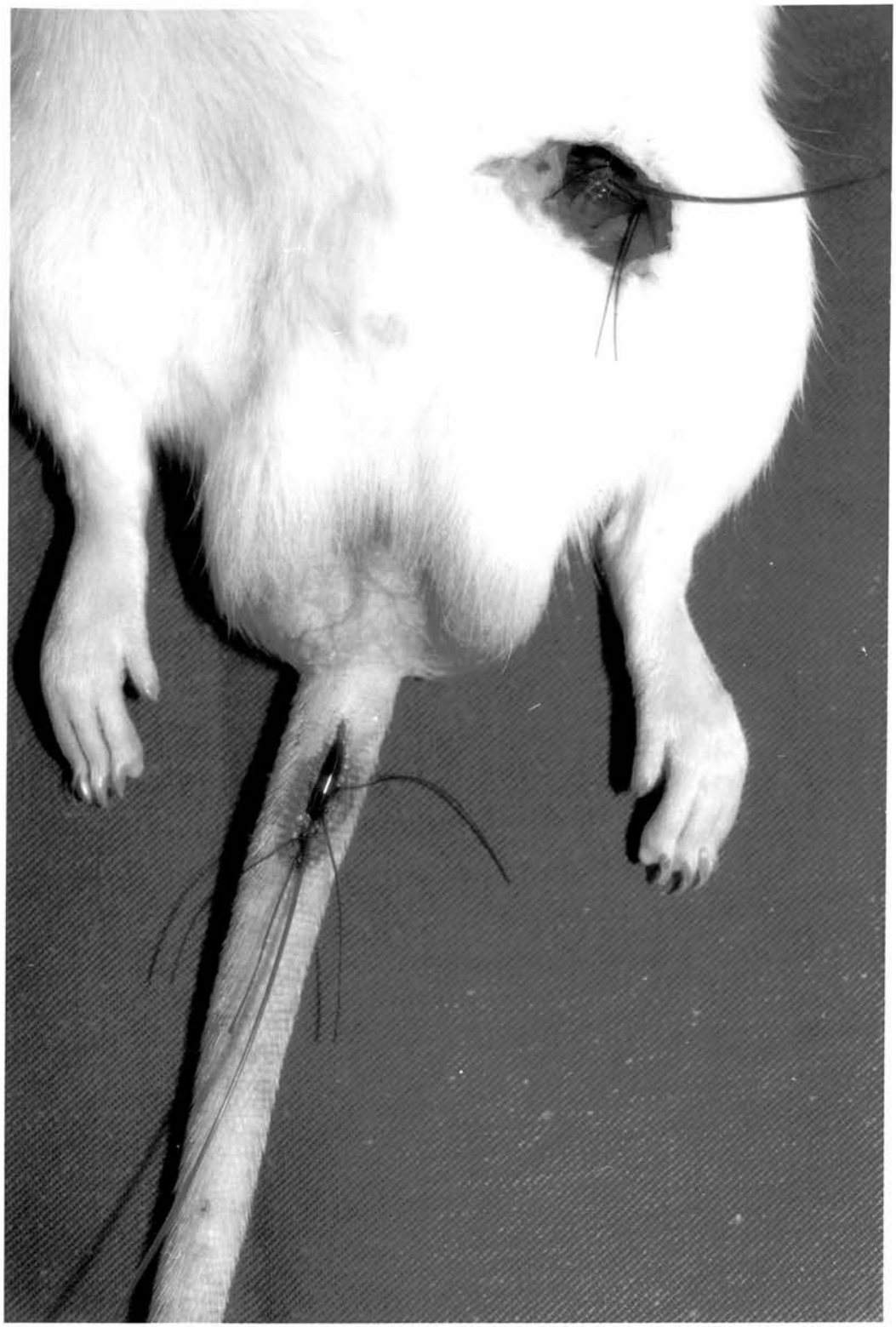
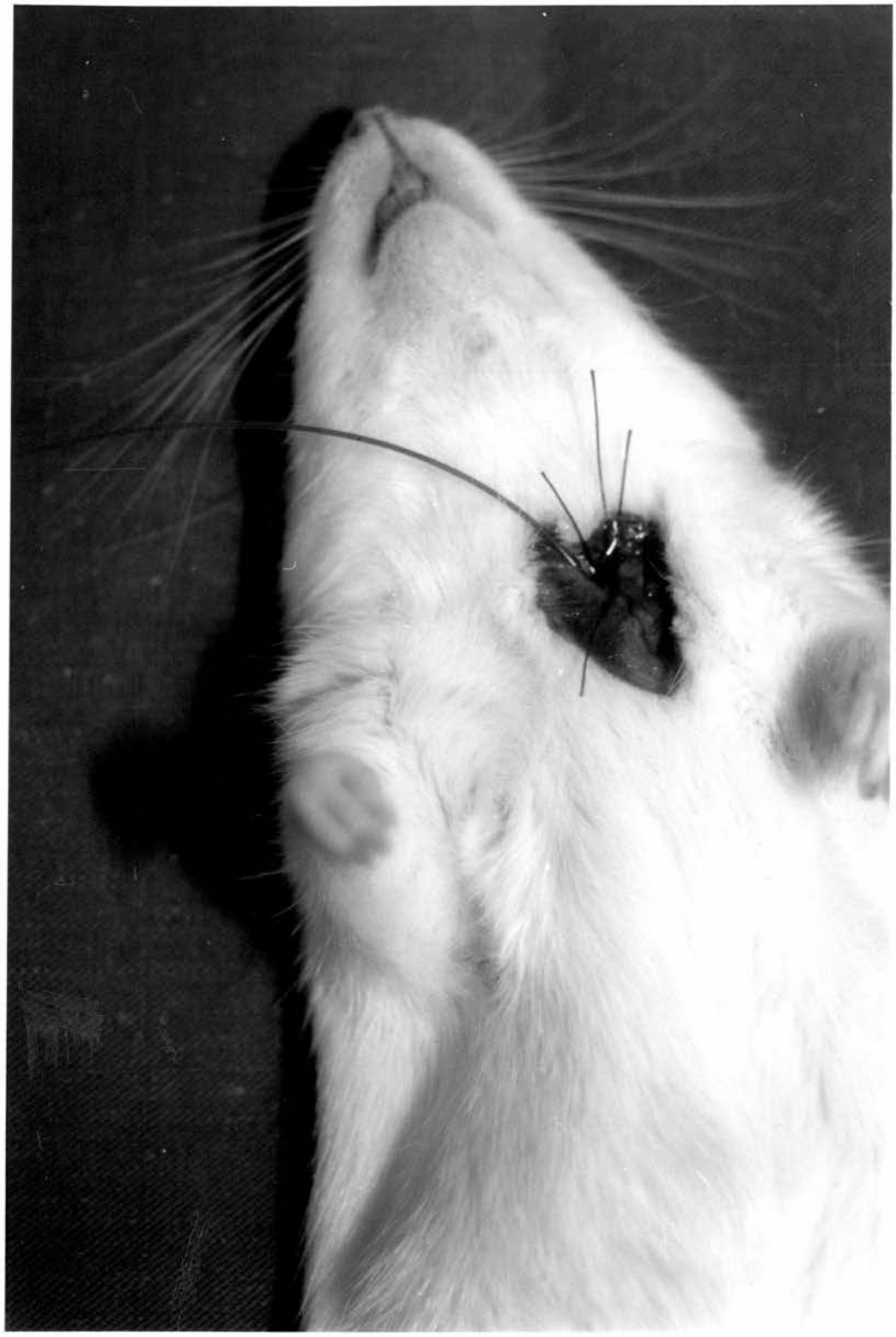


Figure 3.3. Photograph showing cannulation of the carotid artery



The samples were then prepared for counting. The bones were stripped of all muscle and scraped with a scalpel blade thereby ensuring all tissue was removed and so also removing the periosteum. These were weighed and placed in a test tube with a small quantity of formalin.

The muscle sample was weighed and treated in a similar manner. The syringes containing the blood samples were also weighed in order to calculate the actual withdrawal rate.

These samples were then counted on a Wallac L.K.B. gamma counter for 300 seconds. Standards of the isotopes were used to determine the crossover factors. The long half-lives of the isotopes meant that no allowance had to be made for decay in the count period.

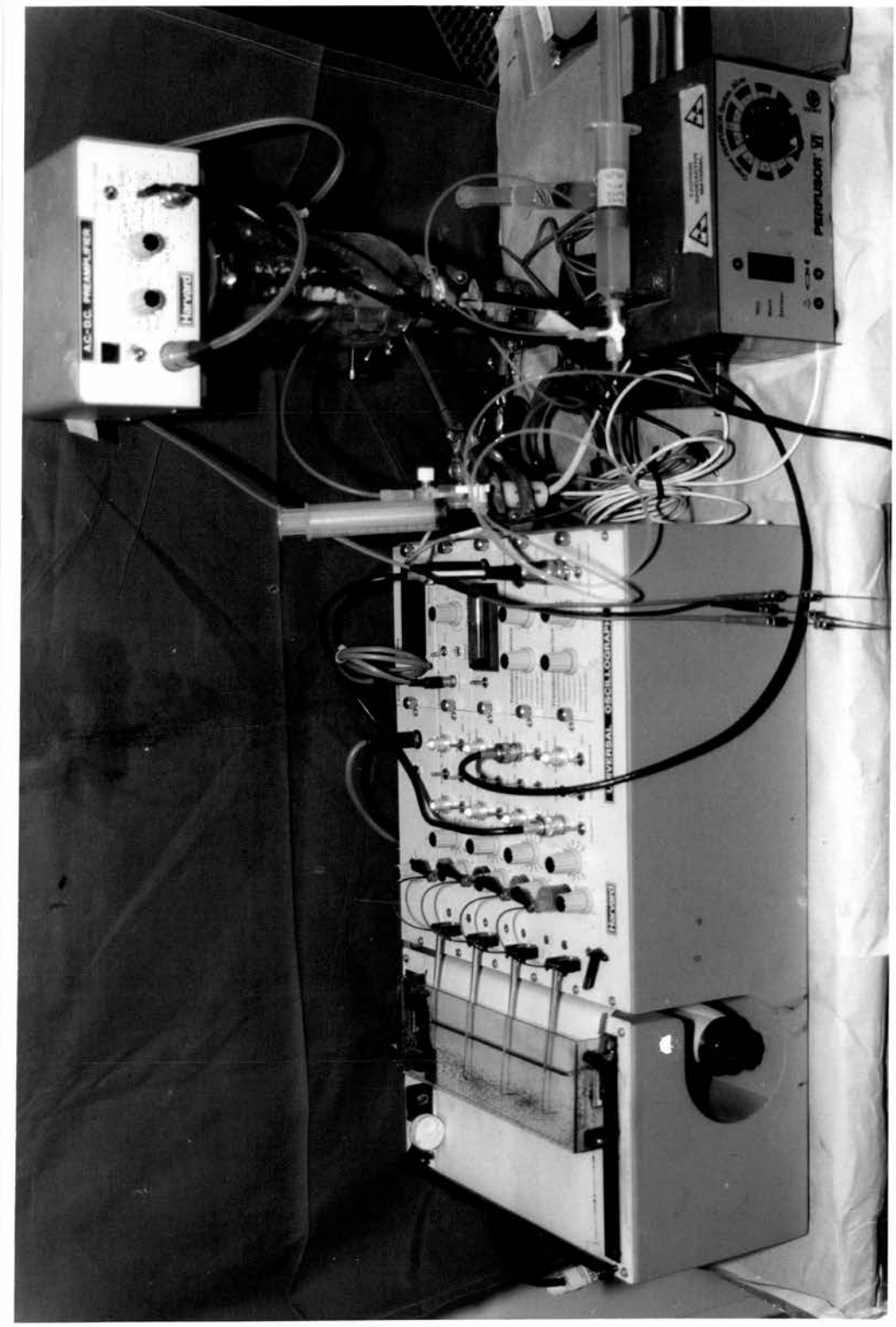
3.2.3. Measurement of arterial blood pressure

A measurement of arterial pressure was made in each animal. This involved connecting a Harvard Physiological recorder (Figure 3.4.) to the carotid cannula via a three way tap. This tap was connected to a second tap on the syringe used for infusion (on the infusion pump). Therefore the carotid cannula was connected to an infusion pump and a pressure recorder.

By measuring arterial pressure constantly any effect of the agents under investigation on blood pressure could be seen immediately and quantified after the experiment by examining the traces provided. This allowed a measurement of change in pressure to be calculated.

Before the experimental procedures the Harvard recording equipment was calibrated. A sealed bottle connected to a pressure transducer (Elecomatic) via a three way tap was pressurised using a Sphygmomanometer. The minimum pressure was then established and depending on the agent used this was set at 40mmHg (vasodilators) and 60mmHg (vasoconstrictors). From other workers the mean arterial pressure was approximately 80mmHg and these two settings were chosen to allow the pressure to decrease or increase from this level and still remain on the trace. The pressure was then increased to the maximum which represented 100mmHg above the minimum i.e. 140mmHg and 160mmHg and the highest point set. After these were set the 0.1V calibration button was used to give a fixed reference sign; this remained constant even when the pen zero was adjusted to increase or decrease the minimum or maximum point. Thus if the pressure trace started to rise above the maximum setting, the pen zero was adjusted to maintain a constant reading and 0.1V button used to re-calibrate the system.

Figure 3.4. Photograph showing the Harvard Physiological Recorder



3.3. Method of calculation

a) Tissue flow rate, strontium clearance and extraction ratio.

Crossover occurs when two or more isotopes of different energies are present in the same sample, in this case cobalt-57, tin-113 and strontium-85. For each standard a spectrum plot, a plot of the energy against intensity of emissions, was produced (Figures 3.5,3.6,3.7). From these the optimum windows were calculated. These represent the windows which minimises the crossover from one isotope into the other while maximising the percentage of total counts within the window (level number).

Table 3.1. Windows selected and % of total counts

	Window	KeV	% of counts
Cobalt	115-135	122	85%
Tin	172-184	392	23%
Strontium	186-198	514	55%

The setting of these windows reduced the spillover of the lower energies into the higher energy tracer. Cobalt-57 has a low energy and the percentage of counts spilling over into the strontium window is less than 0.02% when the standards are used. This was ignored when estimating the strontium activity of the samples thereby simplifying the calculations. Approximately 0.06% of the counts from the tin standard spectrum plot are found in the window selected for strontium. This small spillover of tin into the strontium channel was also ignored when estimating the strontium tissue activity.

Although the spillover from tin into cobalt window is low, less than 3%, this is sufficient to require a spillover factor. Similarly the strontium spillover into cobalt (7%) and tin (4.8%) also requires calculation of a spillover factors. These were calculated using the following equations. The calculations assume that there is no spillover up from the lower energy tracer into the window for the higher energy.

Figure 3.5. Spectrum plot of 57-Cobalt standard

LEVEL NO	% OT	CPM	0 195000 390000 585000 780000
035-040	0.01	151	I
040-045	0.01	95	I
045-050	0.01	189	I
050-055	0.02	322	I
055-060	0.04	596	I
060-065	0.04	682	I
065-070	0.03	578	I
070-075	0.03	512	I
075-080	0.03	578	I
080-085	0.05	872	I
085-090	0.06	946	I
090-095	0.09	1560	I
095-100	0.21	3499	I
100-105	0.67	11149	I*
105-110	1.95	32152	I-*
110-115	2.37	39121	I-*
115-120	7.29	120399	I------*
120-125	47.21	780187	I----------*
125-130	31.18	515272	I----------*
130-135	4.75	78479	I----*
135-140	0.57	9413	I*
140-145	0.55	9063	I*
145-150	0.67	11011	I*
150-155	0.89	14720	I*
155-160	0.94	15505	I*
160-165	0.19	3177	I
165-170	0.02	322	I
170-175	0.03	492	I
175-180	0.01	236	I
180-185	0.01	104	I
185-190	0.01	142	I
190-195	0.01	113	I
195-200	0.01	95	I
200-205	0.02	388	I
205-210	0.03	463	I
210-215	0.00	19	I
215-220	0.00	9	I
220-225	0.00	19	I
225-230	0.00	19	I
230-235	0.00	9	I
235-240	0.00	9	I
240-245	0.00	0	I
035-245	100.00	1652667	

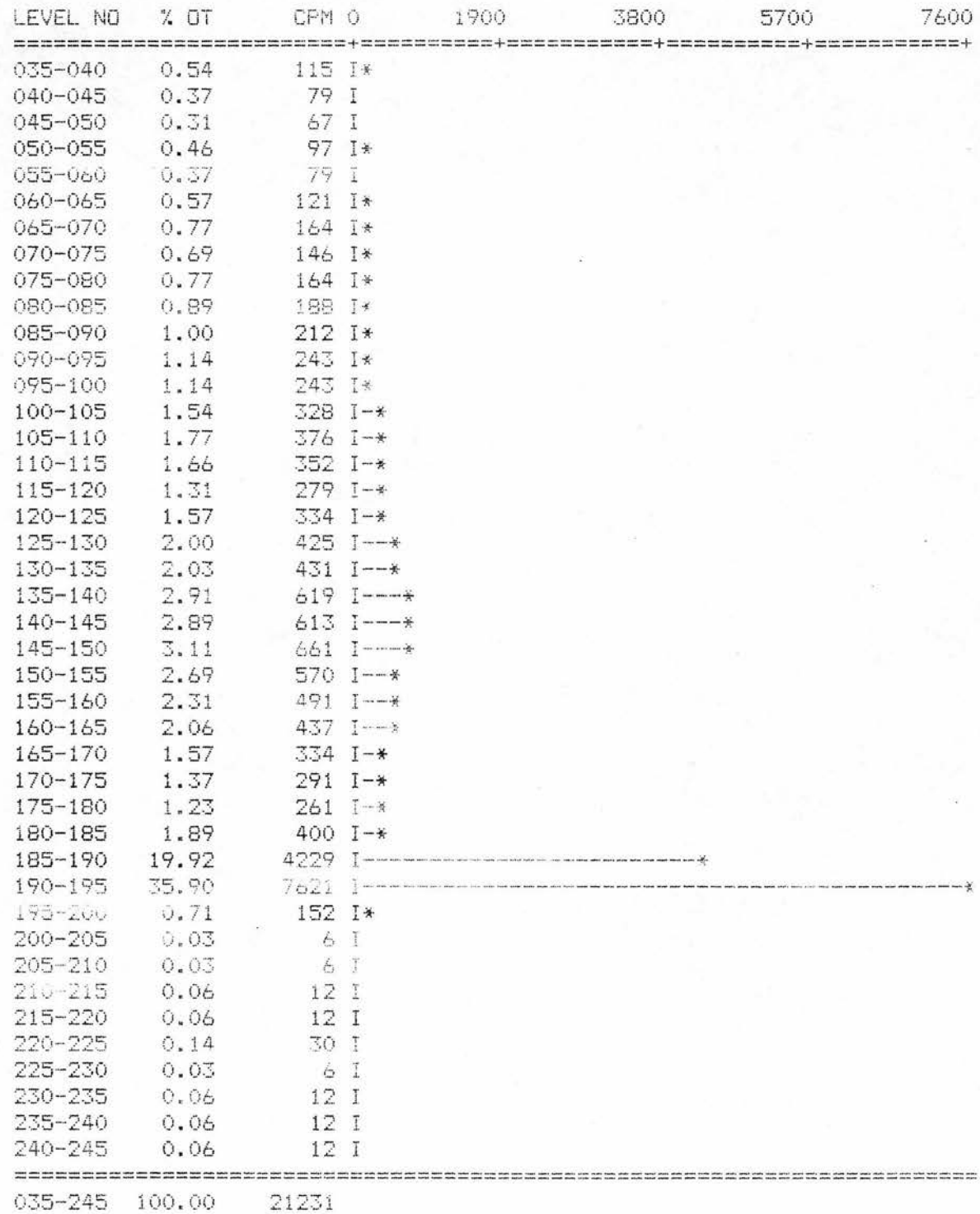
Selected window 115-135 represents 85% of total counts

Figure 3.6. Spectrum plot of 113-Tin standard

LEVEL NO	% OT	CPM	0 17100 34200 51300 68400
035-040	3.75	11799	I--------*
040-045	12.98	40791	I----------------------------*
045-050	21.84	68652	I--*
050-055	15.06	47326	I-------------------------------*
055-060	4.45	13970	I---------*
060-065	0.74	2336	I-*
065-070	0.34	1073	I*
070-075	0.34	1060	I*
075-080	0.38	1204	I*
080-085	0.40	1250	I*
085-090	0.36	1118	I*
090-095	0.38	1204	I*
095-100	0.43	1342	I*
100-105	0.45	1427	I*
105-110	0.43	1355	I*
110-115	0.48	1521	I*
115-120	0.50	1567	I*
120-125	0.50	1587	I*
125-130	0.59	1863	I*
130-135	0.80	2522	I-*
135-140	1.11	3476	I-*
140-145	0.87	2739	I-*
145-150	0.71	2245	I-*
150-155	0.58	1830	I*
155-160	1.03	3226	I-*
160-165	1.10	3455	I-*
165-170	0.68	2152	I*
170-175	2.79	8773	I------*
175-180	20.96	65863	I--*
180-185	4.76	14960	I----------*
185-190	0.05	145	I
190-195	0.03	99	I
195-200	0.02	66	I
200-205	0.02	59	I
205-210	0.01	39	I
210-215	0.02	66	I
215-220	0.01	46	I
220-225	0.00	7	I
225-230	0.00	13	I
230-235	0.01	26	I
235-240	0.01	20	I
240-245	0.00	7	I
035-245	100.00	314278	

Selected window 172-184 represents 23% of total counts

Figure 3.7. Spectrum plot of 85-Strontium standard



LEVEL NO	% GT	CPM	0 1900 3800 5700 7600
035-040	0.54	115	I*
040-045	0.37	79	I
045-050	0.31	67	I
050-055	0.46	97	I*
055-060	0.37	79	I
060-065	0.57	121	I*
065-070	0.77	164	I*
070-075	0.69	146	I*
075-080	0.77	164	I*
080-085	0.89	188	I*
085-090	1.00	212	I*
090-095	1.14	243	I*
095-100	1.14	243	I*
100-105	1.54	328	I-*
105-110	1.77	376	I-*
110-115	1.66	352	I-*
115-120	1.31	279	I-*
120-125	1.57	334	I-*
125-130	2.00	425	I--*
130-135	2.03	431	I--*
135-140	2.91	619	I---*
140-145	2.89	613	I---*
145-150	3.11	661	I----*
150-155	2.69	570	I--*
155-160	2.31	491	I--*
160-165	2.06	437	I--*
165-170	1.57	334	I-*
170-175	1.37	291	I-*
175-180	1.23	261	I-*
180-185	1.89	400	I-*
185-190	19.92	4229	I----------*
190-195	35.90	7621	I----------*
195-200	0.71	152	I*
200-205	0.03	6	I
205-210	0.03	6	I
210-215	0.06	12	I
215-220	0.06	12	I
220-225	0.14	30	I
225-230	0.03	6	I
230-235	0.06	12	I
235-240	0.06	12	I
240-245	0.06	12	I
035-245	100.00	21231	

Selected window 186-200 represents 55% of total counts

i. Spillover factor 1 for strontium into the tin channel

$$\frac{\text{Sr standard counts in the Tin channel}}{\text{Sr standard counts in the Sr channel}} \qquad (10)$$

ii. Spillover factor 2 for strontium into cobalt channel

$$\frac{\text{Sr standard counts in the Cobalt channel}}{\text{Sr standard counts in the Sr channel}} \qquad (11)$$

iii. Spillover factor 3 for tin into cobalt channel

$$\frac{\text{Sn standard counts in the cobalt channel}}{\text{Sn standard count in the Sn channel}} \qquad (12)$$

Examples of these spillover factors are;

1. Strontium into tin - 0.06
2. Strontium into cobalt - 0.13
3. Tin into cobalt - 0.084

These spillover factors are then used in the calculation of true count for the tissue and blood samples.

1. Strontium

True count = sample count - background (13)

2. Tin

True Count = sample count - background - (Sr spillover factor 1 x Sr count) (14)

3.Cobalt

A = sample count - background

B = (Sr spillover factor 2 x Sr count)

C = (Sn spillover factor 3 x True count Tin)

True Co Count = A - B - C (15)

The cobalt blood sample is obtained before the other isotope are added therefore there is no spillover from the other sources. Thus calculation of true blood count is simplified.

1. Strontium and 3. Cobalt

True blood = sample count - background (13)

2. Tin

True blood = count - background - (spillover factor 1 x Sr count)

(16)

In each case the tissue flow can now be calculated using the following equation:

$$\text{BLOOD FLOW} = \frac{\text{True Microsphere count in tissue x pump rate (ml/min)}}{\text{True microsphere count blood}}$$

(17)

These results are then divided by weight of tissue to give values in ml/minute/g.

The removal of strontium from blood during its passage through bone is defined in terms of clearance.

The exact relationship between clearance and flow can be explained in algebraic terms, bringing in the additional variable of extraction.

e.g. If Ca(t) = Quantity entering the system with time

and Cv(t) = Quantity leaving the system with time

then Extraction = $\frac{\int Ca - \int Cv}{\int Ca}$

Then letting the change in quantity = dM

and the change in time = dt

with flow represented by F. a change in quantity overtime can be described as

$$\frac{dM}{dt} = F.\int Ca\,(t) - \int F.\ Cv(t)$$

therefore $M = F.\ [\int_{\circ}^{T} Ca(t)dt - \int_{\circ}^{T} Cv(t)dt]$ at time T

Thus clearance, which is the quantity not deposited in the tissue over the time period of the experiment

ie. Total Quantity (M)

Quantity entering the system with time [Ca(t)dt]

$$= F.\frac{\int Ca - \int Cv}{\int Ca}$$

Thus Clearance equals Flow times Extraction

Strontium clearance can be calculated using the following equation:

$$\text{CLEARANCE} = \frac{\text{True Sr activity in sample x pump rate (ml/min)}}{\text{True Sr activity in blood}} \quad (18)$$

This value for clearance is then divided by the weight of tissue to give the results in the form of ml/min/g.

Strontium-85 can only be partially cleared during its passage through bone and therefore it can be assumed that the value for bone blood flow is greater than that of clearance. The extraction ratio which represents the proportion of strontium deposited is calculated from the values of flow and clearance.

$$\text{Extraction} = \text{Clearance} / \text{Flow rate} \qquad (19)$$

(b) Change in arterial blood pressure.

The arterial blood pressure was measured during the entire procedure, although each animal was connected to a perfusion pump the perfusion did not alter the pressure reading to any extent so this recording was accepted as arterial trace. I was therefore able to note the pressure at specific points in the experiment. These were;

B1 blood pressure at point of cobalt microsphere injection,

B2 blood pressure at beginning of experimental run,

B3 blood pressure at point of tin microsphere injection,

B4 blood pressure at point of maximum drug effect.

Therefore the extent of the action of the administered agents on pressure could be established. The change in arterial pressure as a result of any treatment is calculated from B2 and B4.

$$\text{i.e. Change in blood pressure} = B4 - B2. \qquad (20)$$

In some cases B3 and B4 are the same value so this one value is used in the calculation of the change in blood pressure and the vascular resistance during the Tin blood flow measurements.

In the untreated animals i.e. controls the the values for B1, B2, B3 and B4 are all similar

and no statistical difference is found. However for some of the experimental animals the difference between B2 and B3 is significant as is the difference between B2 and B4. In all cases B1 and B2 are similar, but for the calculation of pressure change the value at the beginning of the experimental run is used to ensure that the administration of cobalt microspheres has no direct effect on the arterial pressure.

(c) Vascular resistance.

Vascular resistance can be defined as the impediment of blood flow through a given portion of the circulation. To calculate it the formula shown below is used;

$$V.R. = \frac{\text{arterial blood pressure - venous blood pressure}}{\text{Blood flow}} \qquad (21)$$

In practice the venous blood pressure from the systemic circulation is so small in relation to the mean arterial pressure that this can be disregarded and the mean arterial pressure used as the numerator of the equation (Marshal et al 1962). Thus the equation becomes;

$$V.R. = \frac{\text{Arterial blood pressure}}{\text{Tissue blood flow}} \qquad (22)$$

In each animal we have a value for both and therefore this can be calculated for both bone and muscle.

3.4. Statistical analysis

The method of analysis used throughout this document are:

1 the Student t-test; paired for flow, pressure and vascular resistance change and unpaired used to test for significant differences from control values,

2. regression analysis (X variable i.e. drug dose is fixed) to test the significance of trends in bone blood flow etc. This identifies the correlation between the concentration of drug and the variable examined. A form of the t-test was then used to see if the slopes of regression are significantly different from one another.

For example, the regression lines for :

1 Control Animals -- dose versus strontium clearance.

2 PGE2 "" ""

i.e

$$t = \frac{b1 - b2}{\{\text{var } b1 - b2\}} \quad \text{on } (n1 + n2) - 4 \text{ D.F.} \qquad (23)$$

Where b = the slope of the line of regression and the standard error of the slope is the square root of its variance.

3. correlation (X and Y variables are random), thus viewing the relation of one variable to the other.

In the case of the paired t-test i.e. for flow, arterial pressure and vascular resistance change, the assumption is that a correlation exists between pre and post treated measurements. The magnitude of this change dependent upon the concentration of administered drug not the initial baseline. Thus animals within any dose group should exhibit changes of the same degree irrespective of the base line and the two measurements should be correlated. The extent of this association is discussed with respect to the control animals in this chapter (3.5.6).

3.5. Development of procedure

This will be divided into 6 sections.

The first section includes the initial study to identify potential problems associated with the technique. These problems are then studied in greater detail in the following sections which attempt to identify the cause and suggest changes to the protocol to rectify the situation. The discussed areas of difficulty include choice of cannulation site, assumed haematocrit values, choice of pump setting and method of randomising the selection of drug and the dose used.

Finally the last section deals with the degree of association between paired measurements with respect to the control animals for the experimental data (i.e. control group 3)

3.5.1. Control animals

Study one ---- Control group 1

An initial study was conducted on eighteen animals to see if the injection of microspheres had an adverse effect on blood pressure and also if a time delay between two measurements of blood flow resulted in any change. The data of two animals are not included because insufficient blood was collected during the first blood flow measurement to establish an estimate of bone blood flow. The results for the remaining sixteen Sprague Dawley rats are presented in Table 3.2. (control group 1).

a). Method

1. The animals were dissected and cannulated as set out in section 3.2.2. This study was set up to view the efficiency of the suggested protocol therefore only the carotid and caudal arteries were cannulated.

2. Measurement of blood flow

The choice, dose and supplier of the microspheres is detailed in section 3.2.1.

In each case two separate measurements of flow were made using two different labelled sets of spheres.Thus for each animal two blood samples were obtained.

Thirty seconds before the microspheres were injected blood withdrawal from the caudal

cannula commenced and this ceased after a total of two minutes (0.197ml/min). Approximately 200 000 cobalt-57 and 113-tin microspheres were injected via the carotid cannula. These injections were separated by about two minutes and during the entire test period the rats were infused with buffer via the carotid cannula at 6ml/hour. This was done to ensure that the cannula remained patent so that the microspheres could be injected and to replace some of the blood lost by the animal. Therefore over each test measurement 0.2ml of buffer was infused.

3. Arterial blood pressure was also monitered during the experimental period, the recorder connected to the animal via a tap attached to the carotid cannula.

4. The animals were sacrificed and both femora and tibia removed and all samples measured for activity.

b). Results

Table 3.2 highlights the variation in the difference between the two flow measurements that can occur in any individual animal, however as a group this difference is low and is not significant (t=1.59 D.F.=13).

The mean values for flow for this group

i.e. Flow 1 Flow 2

0.386 ± 0.193 (SD) 0.305 ± 0.131 (SD)

are higher than those of most other authors (Hruza et al 1969, MacPherson and Tothill 1978, Schoutens et al 1979). This could have arisen through differences in either the technique or the age and species of the animal used.

eg. Hruza - blood clearance measurements using Calcium-45, male and female rats sampling the tibia.

MacPherson - microsphere technique, Wistar rats, 2-17 months old sampling tibia and fibula.

Schoutens - bone plasma flow using red blood cell labelling in female Sprague Dawley rats.

Table 3.2. Control group 1 , infusion of saline over the test period at 6ml/hour (Flow measurements separated by 2 minutes)

	Flow*		#	
Rat no.	57-Co	113-Sn	Sn - Co	Sn/Co
1	0.231	0.287	0.056	1.24
2	0.160	0.087	-0.073	0.54
3	0.121	0.111	-0.01	0.92
4	0.412	0.307	-0.106	0.74
5	0.643	0.357	-0.286	0.55
7	0.579	0.172	-0.407	0.30
9	0.441	0.314	-0.127	0.71
10	0.457	0.254	-0.203	0.56
11	0.131	0.541	0.41	4.**13**
12	0.698	0.522	-0.177	0.75
13	0.462	0.407	-0.055	0.88
14	0.310	0.306	-0.004	0.99
15	0.220	0.271	0.051	1.2
16	0.543	0.328	-0.215	0.6
$\bar{x}$	0.386	0.305	-0.082	1.0
SD	0.193	0.131	0.192	0.94

* Flow is measured in ml/min/g

Students paired t-test, t =1.59 D.F.= 13 Not significant.

$\bar{x}$ = mean. SD = standard deviation

These values are however similar to the top range of the values of Fredrickson et al (1955),who cite a range of 0.1 - 0.3 ml/min/g of tissue for bone blood flow.
The arterial pressure traces during these tests were unaffected by injection of the microspheres, indicating that any effect found after injection of test drug should be due to the test solution.

Study two --- control group 2

A second control group was then studied. Control group 2 consists of eight rats with similar injections of tracer separated by a time period. The procedure was changed to incorporate a 2ml bolus injection of buffer, representing injection of some of the agents. Unfortunatly two rats were of the Wistar variety and these are not included in the results

a). Methods

1. The dissection and cannulation method was the same as the control group 1 animals.
2. Measurement of control blood flow - Cobalt-57 (0.2ml of 20ml saline solution, NEN 0.5mCi)

Thirty seconds after begining withdrawal (0.197ml/min) from the caudal artery Cobalt-57 was injected via the carotid cannula. Blood withdrawal ceased after a total of two minutes.

3. Measurement of test blood flow - Tin-113 (0.2ml of 20ml saline solution, NEN 0.5mCi)

Prior to the second measurement of flow a 2ml bolus of buffer was injected via the carotid cannula, approximately two minutes after stopping the withdrawal for the first measurement. The cannula was reconnected to the infusion pump. Thirty seconds after this withdrawal from the caudal artery was begun. After a further 30 seconds the tin-113 microspheres were administered via the carotid cannula followed by injection of strontium-85. Thus in addition to flow, clearance was measured.

4. The animals were sacrificed and both femora and tibia removed and all samples measured for activity.

b). Results

The difference between the two flows in any particular rat is small apart from numbers 2 and 8, and as a whole this difference is insignificant (Table 3.3.).

The mean values of flow are once again higher than those of the authors cited in study 1 but agree with those of Fredrickson et al (1955).

Thus the evidence shows that a time lapse (approximately 2 minutes) between the two determinations of flow does not result in any loss of accuracy and that any change in flow is caused by the substance injected not the microspheres.

Using the flow and clearance values extraction has been calculated (column 6). All but one value is greater than one. As explained in section 3.3. extraction is an indication of the proportion of strontium deposited and reflects the quantity of strontium that remains in the system i.e. is not cleared by the blood. Extraction is dependant on both flow and clearance and since it is unlikely that all strontium entering the system is deposited, clearance should be less than flow. Here clearance is greater than flow suggesting that strontium is actively deposited. This discrepancy could have arisen through either an underestimation of flow or an overestimation of clearance.

Thus before proceeding further the problem associated with the extraction ratio had to be identified and an attempt made to rectify the situation or a correction factor calculated that could be incorporated into the drug study. The sources of error which could give rise to either an underestimation of flow or overestimation of clearance could arise from the actual microsphere technique. As clearance cannot be greater than flow this would suggest that the problem is related to an underestimation of flow. Therefore a review of the technique was undertaken. Firstly the cannulation sites were considered comparing withdrawl at the femoral and caudal sites. Next the haematocrit of the bone and caudal arteries were investigated to see if either plasma of microsphere streaming occured. Finally the rate of withdrawal was reviewed to see if this was inappropriate, possibly causing the vessel to clamp down and thus not allowing collection of sufficient microspheres.

Table 3.3. Control group 2, injection of buffer as 2ml bolus before second batch of microspheres

	Flow*		#	Strontium	
Rat no.	57-Co	113-Sn	Sn - Co	Clearance *	Extraction
1	0.127	0.266	0.099	0.353	1.56
2	0.199	0.404	0.205	0.316	0.78
3	0.205	0.237	0.032	0.365	1.54
6	0.278	0.297	0.019	0.725	2.44
7	0.443	0.351	-0.092	0.369	1.05
8	0.554	0.286	-0.268	0.65	2.27
$\bar{x}$	0.301	0.3	0.001	0.46	1.61
SD.	0.164	0.07	0.163	0.18	0.65

* Flow is measured in ml/min/g

Students paired t-test , t = 0.01 D.F.= 5 Not significant.

$\bar{x}$ = mean. SD = standard deviation

1. Cannulation site

Study three. -- Withdrawal from the caudal and femoral arteries simultaneously

1. Choice of the caudal artery.

It is accepted that cannulation of the carotid artery allows an entry point into the left ventricle of the heart, providing optimum mixing of the microspheres. The choice of the caudal artery as a reference sampling point is disputed, Brookes (1971) feels that the caudal artery, with its caudal glomeruli is more representative of a mixed supply than an arterial source. Therefore the use of this vessel would not supply an arterial blood sample required for the calculation of bone blood flow.

Although the femoral artery can be cannulated this is not as easy a process as cannulation of the caudal artery. In order to locate the femoral artery extensive dissection of the groin region may be required, while location of the caudal artery only really requires an opening to be made in the upper portion of the tail. Also use of the femoral artery limits the useful bones to only one side meaning that the number of injected microspheres has to be increased to ensure that the two bones contain a sufficient number to allow 95% error limits.

Therefore this section compares the flows obtained when either or both sites are used for withdrawal.

a). Methods

1. The syringes used for withdrawal were weighed prior to the experiment so that the actual withdrawal rate could be calculated.

2. Investigating this, I conducted a series of experiments in which both both the femoral and caudal arteries were cannulated (3.2.).

3. Measurement of flow.

The choice, dose and supplier of the microspheres is detailed in section 3.2.1.

Both cobalt-57 and tin-113 were used to establish bone blood flow measurements. The injection of the two tracers, via the carotid cannula was separated by a few minutes allowing the animal to recover and to establish two flow measurements for comparison. Over the entire test period buffer was infused via the carotid cannula at 6ml/hour .This ensured that the cannula remained patent.Withdrawals of reference blood samples were made from both the femoral and caudal arteries simultaneously (0.197ml/min for two minutes).

4. The animals were sacrificed and both femora and tibia removed and all samples measured for activity.

5. The syringes were weighed after collection and the actual withdrawal rate calculated.

i.e.

$$\text{Withdrawal} = \frac{(\text{Weight 2} - \text{Weight 1}) \ / \ \text{S.G.B.}}{\text{Time of withdrawal}} \qquad (24)$$

S.G.B. = Specific Gravity of Blood -- 1.05

b). Results

There is no significant difference in the flow values obtained from the two sources (Table 3.4.). The mean values for the cobalt measurement is almost identical at the two sources i.e. 0.19 and 0.187 ml/min/g. And although the two femoral measurement are slightly different, i.e.0.05 ml.min/g difference, this is not significant.

Table 3.4. Comparison of blood flow using two sampling sites in the same animal

	Cobalt-57*		Tin -113*	
	Caudal	Femoral	Caudal	Femoral
	0.213	0.164	0.114	0.132
	0.05	0.108	0.05	0.08
	0.19	0.144	0.134	0.198
	0.292	0.344	0.105	0.128
$\bar{x}$	0.187	0.19	0.102	0.134
SD.	0.101	0.105	0.036	0.048

* Flow is measured in ml/min/g

Number of animals (n) = 4.

None of the paired t-tests are significant.

$\bar{x}$ = mean. SD = standard deviation

Study four. -- Withdrawal from the caudal artery followed by dual sampling

The flows estimated of study three (Table 3.4.) are lower than those measured in animals where only the caudal artery has been used for withdrawal (Table 3.2. and 3.3.). This may have arisen because blood has been withdrawn at two sources in study three, possible causing the vessels to clamp down or streaming of the microspheres to occur. In study four the procedure has been changed to allow a comparison between single cannulation and sampling (carotid artery) and dual cannulation with sampling of blood at two sites (carotid and femoral arteries). This should identify if it is the effect of sampling at two reference points that reduces the measured blood flow in study three.

a). Methods

1. The animals were prepared as detailed in section 3.2. cannulating the carotid and caudal arteries. The choice, dose and supplier of the microspheres is detailed in section 3.2.1.

2. The animal was connected to the pressure recorder and infusion pump (6ml/hour) via two three-way taps attatched to the carotid cannula.

3. Blood withdrawal from the caudal cannula was started and after 30 seconds Cobalt-57 microspheres were injected via the carotid cannula. (Withdrawal 0.197ml/ min for 2 minutes).

4. The femoral artery was then cannulated and a second flow rate established using tin-113 microspheres. The blood was sampled at both the caudal and femoral cannula simultaneously, the withdrawal begining thirty seconds before injecting the microspheres (0.197ml/min for two minutes).

5. The blood samples were weighed to calculate the actual withdrawal rates.

b). Results

Withdrawal from the caudal artery has been compared with withdrawal at both femoral and caudal sites during run two in Table 3.5.. Although there is a change in the two caudal flow measurements this is not significant (students t-test). Statistical analysis of the mean difference in the two tin measurements i.e. Caudal 0.707 and Femoral 0.567 shows that there is no significant difference.

Thus there is no difference in the flow estimates between the two sites (femoral and caudal) or between the runs (cobalt and tin).

These flow estimates are well above the values for the previous study i.e. over 0.5 compared with 0.29 (maximum value in Table 3.4). These are also higher than the values of the control group, being almost double the mean values.

Each study represented animals chosen from separate sibling groups i.e. control group 1 represented one group of sibling animals, control group two a second sibling group etc. This therefore suggests that there is a large variation in animals between separate sibling groups which could result in these different flow measurement groups. The variation (standard deviation) with in any group is low indicating that the flow measurements are acceptable. Thus a review of the selection of rats was required to examine this with in group variation compared with the between group variation. The study is presented in a further section.

Table 3.5. Withdrawal from the caudal artery (run 1) compared with withdrawal from the caudal and femoral arteries (run 2)

	Caudal*		Femoral *	(Tin)
	Cobalt	Tin	Tin	Fem - Cau
	0.904	1.007	0.986	-0.021
	1.364	0.860	0.613	-0.247
	0.373	0.522	0.372	-0.15
	0.208	0.606	0.397	-0.209
	0.465	0.502	0.569	0.067
	0.440	0.743	0.466	-0.277
$\bar{x}$	0.626	0.707	0.567	-0.14
SD.	0.429	0.201	0.225	0.139

* Flow is measured in ml/min/g

Number of animals (n) = 6.

None of the paired t-tests are significant.

$\bar{x}$ = mean. SD = standard deviation

2. Assumed haematocrit

Study 5. Measurement of bone and caudal haematocrit

Assuming that the caudal artery is an adequate source of arterial blood, the next question is whether the haematocrit of the reference sample is the same asthe bone haematocrit. In the control animals it is assumed that the labelled blood flowing to bone has a similar microsphere concentration to the blood collected. If this is not the case then this could result in the under or over estimation of bone blood flow.

Blood flow and blood volume are easy to define and possible to measure. The measurement of tissue haematocrit involves the principle of dilution analysis. The most practical method of measurement of red cell volume involves the use of radionuclides, labelling a small volume of the animal's blood cells with either radioactive chromium, technetium (pertechnate) or indium. From the dilution of the labelled cells in the circulation of the animal the red cell volume can be calculated. Using a similar procedure the plasma volume can be measured directly by injecting 125-I labelled albumin. A measurement of red cell and plasma volume can be made in the same animal and the haematocrit of a selected circulation can then be calculated.

The use of red blood cell and plasma labelling allows a comparison of the haematocrit of bone and caudal artery to be made. Therefore the results can be assessed to see if a correction factor for either blood flow or strontium clearance is required.

a) Methods and materials

1. The animal was anaesthetised and the carotid and tail arteries cannulated following the procedure set out in 3.2.2.

2. Red blood cell and plasma labelling

(a) I-125 labelled Albumin and Technetium (Amerscan Stannous Agent)

A 0.01ml solution of stannous salts was injected via the tail cannula (4.0mg stannous fluoride and 6.8mg sodium medronate in 6ml saline). Twenty to thirty minutes after the

injection of the stannous salts a 1ml blood sample was withdrawn from the tail artery. This sample was incubated with technetium (10-13mCi sterile eluate from Technetium-99m generator) for ten minutes. Next the sample was centrifuged at 500g for ten minutes, the supernatant removed and the cells resuspended in saline. This was then injected back into the rat via the carotid cannula along with I-125 labelled albumin (human labelled albumin, Amersham).

(b) Chromium, Indium and I-125 labelled Albumin (Amersham)

A 1ml sample of blood was withdrawn and to this 0.15ml of acid citrate dextrose solution (ACD) was added, this was made up to 15ml using saline and the solution centrifuged to sediment the red blood cells (1000g for 5 minutes). The supernatant was then removed and type cells resuspended in saline. One drop of indium oxine-111 (sterile isotonic aqueous solution of indium oxine complex) and three drops of 51-Cr chromate (sterile solution of sodium chromate in isotonic solution) were added to this and the mixture incubated at room temperature for 15 minutes. A further 5ml of saline was added to wash the cells before a second centrifuging, the supernatant was once again removed and the cells resuspended in 1ml saline. This was then injected via the carotid cannula into the rat at the same time as the 125-I labelled albumin.

(a) and (b)

After two minutes, to allow the red blood cells to mix in the circulation, withdrawal of blood from the tail cannula commenced. Ceasing after two minutes.(0.197ml/minute.)

The animal was then sacrificed and the femora and tibiae removed. After the collected blood sample was centrifuged (5 minute 3600rpm) the volume of supernatant and red blood cells was estimated, the supernatant removed and the samples then analysed for radioactivity.

The energies of each label used determines the settings for each window, (level number) each is set to minimise the amount of spillover between isotopes.

Label	Window	KeV
25-I	30 - 75	35
99mTc	100 - 140	140
51-Cr	160 - 180	320
111-In	170 - 190	390

b). Method of calculation

For each animal we have a value for :

(1) Bone weight.

(2) Bone count (99-Tc or 111-In and 51-Cr. 125-I).

(3) Blood sample weight (x specific density of 1.05, to give blood volume).

(4) Blood count (99-Tc or 111-In and 51-Cr. 125-I).

(5) Measured volume of sedimented blood cells.

(6) Measured volume of centrifuged blood sample.

and using an approximate value for tail haematocrit of 45% (Brookes 1965) we can calculate the bone haematocrit for the experimental animals.

Technetium and I-125 labelled animals.

The spillover of technetium into the iodine counts was small because there was a two day delay in counting for 125-I to allow the technetium to decay.

From each of the counts the background is subtracted.

$$\text{True Tail Haematocrit} = \frac{\text{Measured volume of R.B.C.}}{\text{Measured total volume.}} \quad (25)$$

$$\text{Volume of R.B.C.} = \frac{\text{Bone 111-In}}{\text{Blood 111-In}} \times (\text{blood volume} \times 0.45) \quad (26)$$

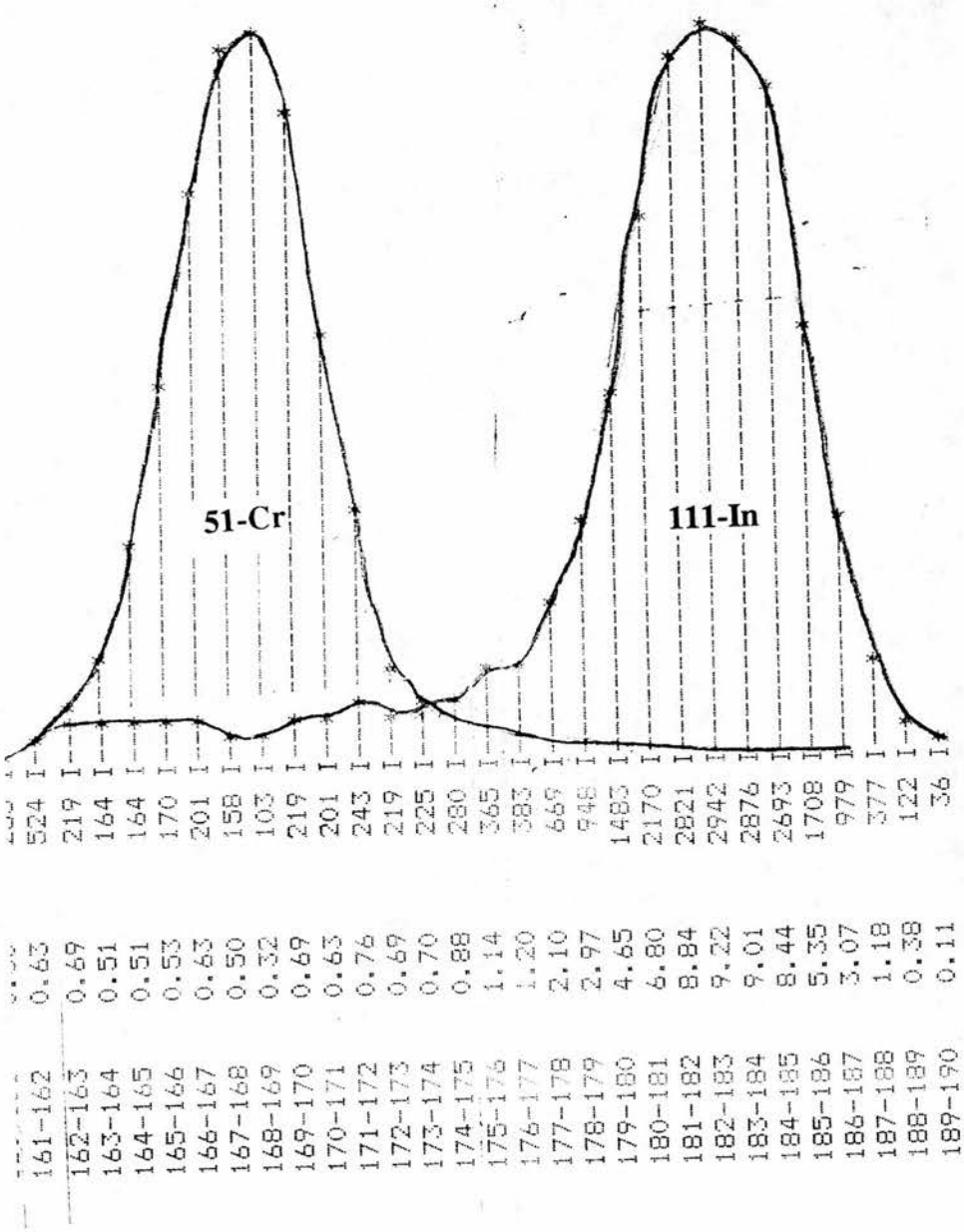
$$\text{Volume of Plasma} = \frac{\text{Bone 125-I}}{\text{Blood 125-I}} \times (\text{blood volume} \times 0.55) \quad (27)$$

$$\text{True Bone Haematocrit} = \frac{\text{Volume of R.B.C.}}{(\text{Volume of R.B.C.} + \text{Volume of Plasma})} \times 100 \quad (28)$$

Chromium, Indium and 125-I labelled albumen.

This presents more complicated calculations since the 111-In and 57-Cr not only spillover into 125-I but into each other (Figure 3.8.).

Figure 3.8. Diagram showing the overlapping spectrum plots of 111-In and 51-Cr



Thus four spillover factors must be calculated before the true counts for the samples can be obtained ;

1. 51-Cr spillover into 111-In (i)

i.e. 51-Cr standard in 111-In channel

51-Cr standard in 51-Cr channel

2. 111-In spillover into 51-Cr (ii)

i.e. 111-In standard in 51-Cr channel

111-In standard counts in 111-In channel

3. 51-Cr spillover into 125-I (iii)

i.e. 125-I standard in 51-Cr channel

51-Cr standard in 51-Cr channel

4. 111-In spillover into 125-I (iv)

i.e. 125-I standard in 111-In channel

111-In standard in 111-In channel

Calculation of true chromium and indium counts

For the purpose of the calculation ;

let X = 111-In counts in A

let Y = 51-Cr counts in B

Thus $X + (i)Y$ = Measured 111-In counts (29)

$(ii)X + Y$ = Measured 51-Cr counts (30)

If equation (30) is multiplied by the spillover factor (i) (31)

We can calculate true X by subtracting this new equation from equation (29).

Thus

$$Y = \frac{(29) - X}{(i)} \quad (32)$$

Calculation of true Iodine counts.

True count = I-125 counts - (Y x (iii)) -(X x (iv)) (33)

Once these ''true count'' values have been calculated the haematocrit of bone and tail can be calculated as for technetium.

c). Results

The use of three separate red blood cell labels provide different estimates for the bone haematocrit, ranging from 47 to 100 % of the assumed arterial haematocrit. The actual values and the associated mean and standard deviations are shown in Table 3.6. The haematocrit using 99-Tc differs significantly from the means when the other labels are used ($p<0.02$ d.f.=4). Similarly the plasma and erythrocyte volumes for the three labels differ, the 99-TC label giving higher estimates in both cases although in neither case are these differences significant.

In the table all values are calculated using an assumed arterial haematocrit of 45%. This is obtained from the work of Brookes(1965). However the measured arterial haematocrit varied from 40-59% in the four animals where the tail haematocrit was calculated. This represents only two measurements of tail haematocrit for each technique and the

range may result from variation between animals or experimental variation. The study does not allow this to be fully investigated or any statistical analysis to be made. If the range does reflect natural variation in the haematocrit between animals this will affect, to some extent, the variation in the tissue haematocrit. Thus the use of an assumed haematocrit of 45% would incorporate a degree of variation into the calculation of bone and caudal haematocrit values. The use of actual measured haematocrits is considered in the discussion but because of the degree of variation in the measured values there are problems in the calculation of any correction factor.

d). Conclusions

This work therefore points to a difference in the haematocrit of bone and the caudal artery. However the actual extent of this difference cannot be successfully estimated from this study because of the extent of variation and lack of animals. The possible causes of this difference are assessed later in this chapter, including evidence that supports the suggestion that bone is richer in plasma and poorer in blood corpuscles than the caudal blood. This would cause a decrease in the microphores entering the bone while increasing the strontium portion of labelled blood, thus giving rise to an under estimation of flow or overestimation of clearance.

Table 3.6. The distribution of three blood cell labels and 125-I labelled albumin as a plasma indicator in rat bone, assuming an arterial haematocrit of 45%

	Volume (ml/100g)		Haematocrit R.B.C %	
Technique	Plasma	Erythrocyte	Bone	Tail
99-TC	3.89	1.23	24	/
	2.17	0.58	21	/
	3.56	1.13	24	51
	2.71	1.07	28	59
n = 4	3.08 ± 0.78	1.0 ± 0.3	24 ± 3	
111-In		0.55	45	
		1.07	37	
n = 2		0.81 ± 0.3	41 ± 6	
	* 0.67			40
	1.89			43
	1.3 ± 0.86			49 ± 7
51-Cr		0.35	34	
		1.10	37	
n = 2		0.72 ± 0.5	35 ± 2	

Mean ± standard deviation

* Two techniques were carried out simultaneously on the same rat thus plasma volume and tail haematocrit are the same for the two procedures.

3. Pump setting.

Next I investigated the effects of the pump rate on flow withdrawal.

Study six. -- Withdrawal rate

a). Methods

1. The animals were prepared using the same method as for study group 4.

Reviewing the estimates for femoral withdrawal rates of this group of animals it was decided to increase the rate of withdrawal at the femoral cannula to 0.492ml/min which represented the actual rate better.

2. Measurement of blood flow.

Cobalt and tin microspheres were used to measure bone blood, the same method as in the previous studies was used i.e. injection via the carotid cannula with a short time period between the two injections.

Using the femoral as a reference organ and the withdrawal at a constant rate the setting of the caudal pump was varied ie.0.079, 0.197 and 0.393 ml/min.

After collection the blood samples were weighed to calculate the actual flow compared with pump setting. The samples were then analysed for radioactivity and the data then converted into two ratios to allow analysis.

b). Method of calculation

i.

$$\text{Ratio of Flow} = \frac{\text{Measured withdrawal rate Caudal}}{\text{Measured withdrawal rate Femoral}} \quad (34)$$

ii.

$$\text{Ratio of Ms count} = \frac{\text{Caudal sample Ms count}}{\text{Femoral sample Ms count}} \quad (35)$$

c). Results

At none of the pump speeds do the flow and count ratios different drastically (Table 3.7.). The small numbers i.e. three animals in each group negates the use of the paired t-test. However comparing the two ratios within each group it appears that the 0.197 ml/ minute setting provides the most reproducible results, showing the least variation between the two ratios. The ratio of microsphere counts is higher than that for the flow at the lowest setting and since the number of microspheres in the femoral blood is relatively constant in each sample this indicates a high level of microspheres in the caudal blood compared with the femoral, possibly caused by pooling or streaming at this pump setting. The lower ratio for microsphere count found at the 0.393 ml/minute setting could be caused by streaming of the microspheres. At the higher flow rate results suggest that the vessels remains patent during the withdrawal although on numerous separate occasions during other flow measurements the vessel was seen to clamp down. This clamping reduces flow and thus the number of microspheres collecte and makes this withdrawal rate unsuitable.

c). Conclusion

The 0.197ml/min rate appears to be the best withdrawal rate for blood collection, since the results are reproducible as indicated by the low standard deviation value. Although the other two rates have similar standard deviation values there is some indication of streaming of the microspheres and clamping down of the vessel at the higher rate, thus making these settings less suitable.

Table 3.7. Comparison of three pump settings on withdrawal of blood at two sites in three animals

Rat		Calculated Caudal withdrawal	Caudal Flow	: Femoral # Count
*	a	0.09	0.17	0.19
	b	0.107	0.2	0.4
	c	0.092	0.37	0.35
	$\bar{x}$	0.096	0.25	0.31
	SD.	0.009	0.11	0.11
**	a	0.21	0.52	0.58
	b	0.29	0.56	0.55
	c	0.19	0.38	0.35
	$\bar{x}$	0.23	0.498	0.49
	SD.	0.05	0.09	0.125
***	a	0.49	0.82	0.82
	b	0.47	0.9	0.66
	c	0.425	0.82	0.87
	$\bar{x}$	0.46	0.85	0.78
	SD.	0.03	0.05	0.11

\# Femoral withdrawal constant --- 0.492 ml/min.
* Caudal withdrawal constant --- 0.079 ml/min.
** " " " --- 0.197 ml/min.
*** " " " --- 0.393 ml/min.

$\bar{x}$ = mean. SD = standard deviation

4. Administration of drug and experimental variation

The choice of administration was determined by the half life of the drug used and this section reviews the control animals (group 1 and 2) to establish if injection or infusion of the test compounds had any direct effect. The variation caused by selection of animals from one sibling group is then compared with the degree of variation when the animals are chosen at random from more numerous sibling groups.

Studies 1 and 2 Infusion or injection

The procedures followed and results are detailed in section 3.5.1.

Table 3.8. The mean flows and associated standard deviation values for study group 1 and 2

	Flow 1	Flow 2	Number
Control 1	0.386 ± 0.193	0.305 ± 0.131	n=14
Control 2	0.298 ± 0.16	0.3 ± 0.07	n=6

Comparison of the two methods of administration involve only flow 2, as the injection of the bolus occurs thirty seconds before withdrawal for the second flow estimate began. However here both flow 1 and flow 2 (Table 3.8.) are compared using an unpaired t-test. There is no significant difference between the infusion of Krebs Ringer buffer (0.2ml) over the test period or injection of a 2ml bolus following injection (Flow 2 $t = 1.5$ D.F.=18). There is also no significant difference between the two flow 1 means ($t = 0.721$ D.F.=18). Therefore it is acceptable to combine infusion and injection control animals to establish the error parameters associated with this method of measuring blood flow and clearance.This gives a control group of twenty animals.

Table 3.9. Mean flows and standard deviations for the all combined group

	Flow 1	Flow 2	F 2 - F 1
$\bar{x}$	0.36	0.30	-0.06
SD.	0.184	0.114	0.183

Paired t-test t = 0.63, degrees of freedom = 19 Not Significant.

These values are higher than those of Schoutens (1979) and Tothill (1986).

Systemic preference ?

Control Group three ---- Left or right systemic preference

A review of the evidence concerning any systemic increase to one side or the other is of relevance to the haematocrit study where because of cannulation of the femoral artery only one set of femora and tibiae were available for counting. Using control group three (see Section 4, Table 3.11.) the flow values have been calculated in the form of left and right side (Table 3.10.). This table highlights the analysis of the paired data, none of the groupings are significant when a students t-test is used ($p<0.1$). Thus there is no preference for the microspheres to accumulate on either side and this evidence supports the assumption that the microspheres have been adequately mixed in the circulation.

Table 3.10. Comparison of flow to the left and right hind limbs in control group three animals

	Left	Right	L - R
Cobalt	0.33 ± 0.14	0.29 ± 0.13	* 0.03 ± 0.07
Tin	0.37 ± 0.16	0.33 ± 0.16	* 0.04 ± 0.09
Tin - Co	*0.03 ± 0.11	*0.009 ± 0.09	

All values represent mean ± standard deviation.

Number of animals (n) = 20

* paired t-test in all cases $p < 0.1$ i.e. not significant.

Experimental 0 dose animals

The first two control groups (Table 3.8.) were drawn from animals which were delivered in sibling groups, 6 to 8 animals at a time. This would decrease the variation between animals in any one group but would increase variation between groups. Therefore before tackling this study the procedure for selection of animals, drug and dosage was changed. The number of animals were ordered in larger quantities, 16-24 at any one time and in any one day the number used was increased in an attempt to minimise experimental variation. The drug choice was randomised, for example in the case of PTH the 0, 4, 8 and 12μg doses were given in any order to animals from more than one sibling group. Thus the results of any complete drug trial are gathered from animals of different sibling groups in an attempt to minimise the variation between groups, perhaps at the expense of increasing the variation within a group.

This third control group represents all animals given 0 drug, the animals are selected from numerous sibling groups and the total number gathered from experiments performed over a long time (approx 18 months). Using the mean and standard deviations for the flow measurements the variation due to the technique can be calculated (Table 3.11.).

Experimental variation

Before discussing the calculation of experimental variation some knowledge on the types of variation in the above data is essential.

a). Firstly there is the variation within the population i.e. the spread of the values of interest . This will therefore reflect the population variation and thus be free of any differential treatment effect and is therefore a measure of error variance.

i.e. Var (between animals) + Var (technique).

b) From the subtraction of two measurements made with in the same animal i.e. F2 - F1 a value for the experimental variation can be estimated, assuming that the variation between animals has been cancelled.

i.e. Var (technique).

Using these definitions the extent of the variation resulting from the experimental technique can be calculated.

Method of calculating experimental variation (Table 3.11)

1. Variation due to experimental technique.

F2 - F1 = 0.006 ± 0.053

Var (F2 -F1) = Standard deviation^2

= 0.053^2

= 0.0028

= Var (F1 technique) + Var (F2 technique)

Var (technique) of one measurement

= Var (F1 technique) + Var (F2 technique) ÷ 2

= 0.0028 ÷ 2

= 0.0014

Standard deviation (technique) = $\sqrt{0.0014}$

= 0.037

This is the standard deviation associated with the experimental technique for one measurement of blood flow.

2. Variation due to experimental technique and variation between animals.

In this case we have two measurements, F1 and F2, to calculate the mean variance the values are added then divided by two.

i.e. Var F1 = Standard deviation^2 Var F2 = Standard deviation^2

= 0.124^2 = 0.125^2

= 0.015 = 0.016

This is the variation that arises through variation (between animals) and variation in the technique.

Standard deviation = 0.124

$$\text{Fraction of overall error due to tech.} = \frac{\text{Var (technique)} \{1\}}{[\text{Var (between animals)} + \text{Var (technique)}] \{2\}}$$

In this case $0.0014 \div 0.015 = 0.093$

Therefore the error associated with the technique is approximately 9% of the overall error, an acceptable value. Thus increased randomness of animal and drug choice minimises the errors associated with the technique and reduces the variation between groups at the expense of increasing the overall group variation.

The error parameters associated with strontium clearance (Table 3.11.) cannot be calculated in this manner. However the variation in flow caused by the experimental technique should be the upper limit of the variation effect on clearance. The estimates of flow and strontium clearance are measured at the same time. A plot of flow versus clearance demonstrates that a significant relationship between these two variables exists (Figure 3.9.). Strontium clearance appears to vary directly with flow.

Table 3.11. Control animals from the drug treated rats

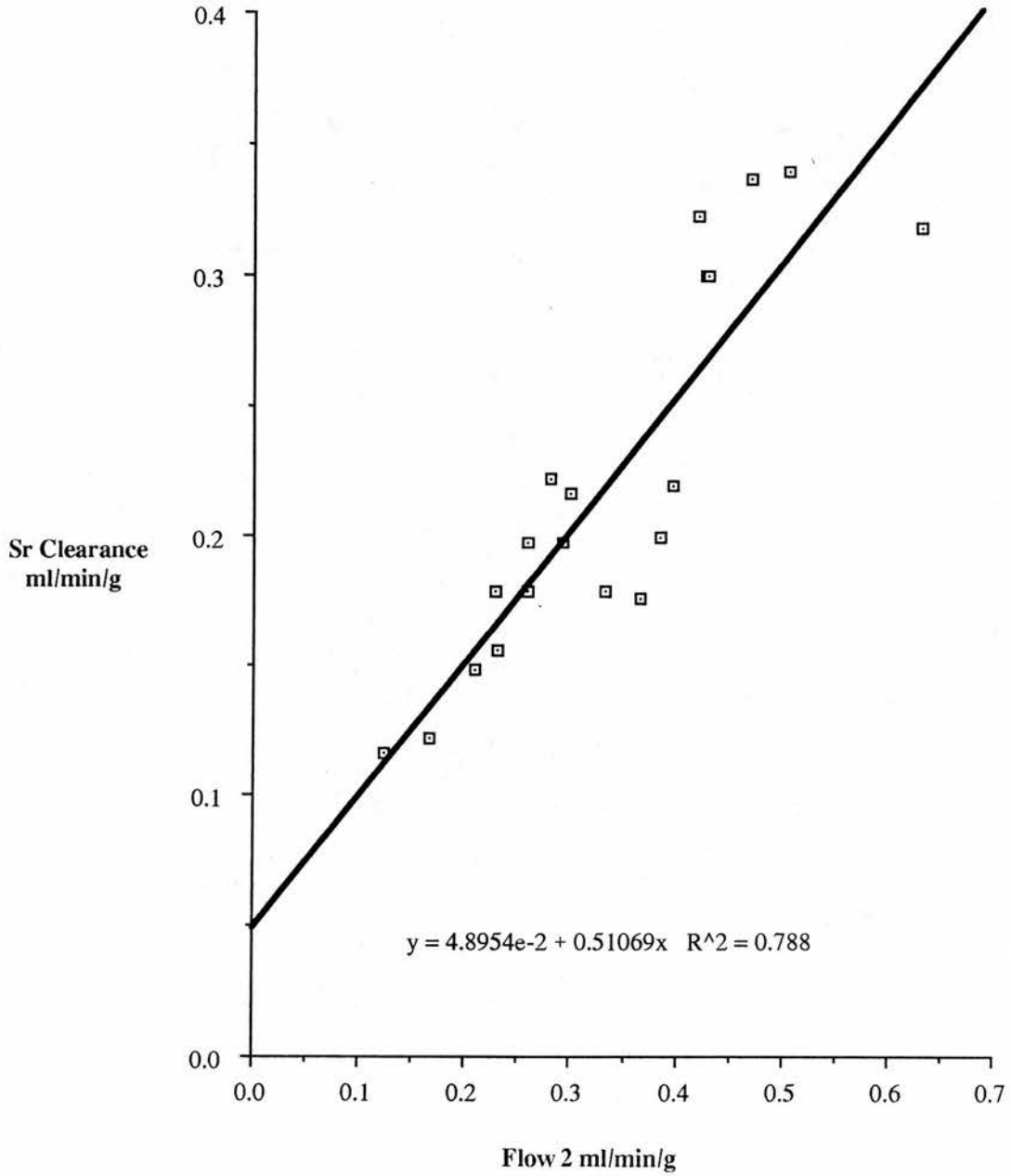
1. Vasoactive Agents

	Flow			Sr Clearance
Drug	*57-Co*	*113-Sn*	*Sn - Co*	
ATP	0.234 ± 0.092	0.22 ± 0.036	-0.014 ± 0.035	0.16 ± 0.05
Nor.	0.352 ± 0.197	0.27 ± 0.07	-0.082 ± 0.175	0.24 ± 0.09
2. Calcium Regulating Hormones				
PTH	0.232 ± 0.098	0.332 ± 0.055	0.087 ± 0.105	0.17 ± 0.02
PGE2	0.49 ± 0.115	0.497 ± 0.098	0.007 ± 0.072	0.20 ± 0.02
Cal.	0.297 ± 0.041	0.215 ± 0.036	-0.102 ± 0.106	0.32 ± 0.02
Combined (n) = 21				
	0.324 ± 0.124	**0.33 ± 0.125**	**0.006 ± 0.053**	**0.24 ± 0.07**

All values represent mean ± standard deviation, with drug referring to the group that the control (0) dose animals are included in.
Flow and clearance are measured in ml/min/g.
Nor. = Noradrenaline. Cal = Calcitonin.

An important point to note in this table is that the mean clearance values are less than the mean flow values in each respective group.

Figure 3.9. Flow 2 Versus Strontium Clearance in the Control Animals



Significance of the regression , $p \leq 0.0001$

Correlation coefficient (r) = 0.89

5. Degree of association between paired measurements

As outlined in section 3.4. the pre and post treated measurements should be related in order to use a paired students t-test.

i.e. Paired data = Blood flow measurements

Arterial pressure

Vascular resistance

The paired data from control group three were treated using regression analysis, in each case the significance of the slope was 0, the correlation coefficient greater than 0.9 and the percentage variation in the second measurement due to its relationship with the first greater than 82%. Thus there is a high association between the two measurements made in the control animals and therefore the use of microspheres to measure flow does not significantly affect this variable. I would assume that this relationship would hold in the experimental animals and therefore any significant difference found would be a result of the drug treatment.

3.6. Discussion

3.6.1. Cannulation site and microsphere technique

1. Carotid site

In order for the microsphere technique to be successful, immediately after injection into the circulation the spheres have to be adequately mixed and evenly distributed in the blood stream. Here the cannulation procedure followed involves insertion of a cannula into the left ventricle. Heyman et al (1977) noted that although the injection should be made into the left atrium, the left ventricle provided accurate measurement of systemic blood flow. Additional support for this method is given by the work of Tøndevold (1983) and Schoutens (1979) who use a similar procedure. Thus injection of the microspheres into the left ventricle should provide adequate mixing and this view is confirmed in the presented results.

2. Caudal site

The choice of caudal artery is queried, although a similar method was used by and Schoutens et al (1979) who cannulated the ''ventral artery'' stating that an arterial sample was withdrawn from the tail. Murray Brookes has pointed out that the rat tail contains ''caudal glomeruli'', arteriovenous anastomoses occurring between the medial caudal artery and the vein. Thus withdrawn blood from the caudal artery could contain mixed blood therefore dilution of the microspheres would arise because of the addition of the venous supply. This dilution however depends on the withdrawal rate and the arterial blood pressure. If the rate of withdrawal is too high then venous blood may enter the vessel to compensate for the sampled arterial quantity, and a lowering of the arterial blood pressure below that of the venous side would allow venous blood to seep into the arterial supply. Kondo (1972) confirmed the presence of these glomeruli. However in establishing the general fine structure this author only mentions the distal third portion of the medial caudal artery as a location. No statement is made to say that glomeruli occur out with this area. The upper portion of the caudal artery may however contain

these . As long as the arterial pressure in the caudal artery is not adversely lowered there may not be any effect even if glomeruli are present. This is supported by the lack of any statistical difference between sampling at the caudal and femoral sources (3.5.2.). Other authors have used the femoral as a withdrawal site (Tothill et al 1980) so if this source of arterial blood is adequate then the caudal artery must give a similar acceptable supply.

3. Microspheres number and evenness of mix

The number of microspheres also affects measurement of blood flow. Buckberg et al (1971) have calculated that 393 microspheres are required in the tissue sample to measure a 10% difference with 95% confidence. Here each injection of radionuclide contained approximately 200 000 microspheres. A level of 400 or more, estimated from the count per sphere made for each microsphere batch, was obtained in all the samples used. This therefore acted as a secondary check on the distribution of the microspheres. The evenness of mixing can be assessed by injecting two sets of microspheres a few minutes apart; the patterns of distribution should be similar for each injection (Heymann et al 1977). Although here I do not have data on the distribution pattern the degree of association between the cobalt and tin measurements (section 3.5.1.) suggest that these are similar. A comparison of flow to left and right hind limbs (3.5.5.) shows that no systemic preference for microsphere collection occurs supporting the view that the microspheres have been sufficiently mixed in the blood thus representing the the actual flow.

4. Microsphere size

The size of the microspheres is important when considering entrapment and arterio-venous shunting. This method of measuring blood flow assumes that the microspheres entrap completely in one passage but do not have haemodynamic effects. Few studies however have investigated this complete entrapment with regard to bone.

Gross et al (1979) evaluated arteriovenous shunting in bone blood vessels using different sized microspheres. Blood flows measured using 7-10μm diameter spheres were 35% lower than those made with 15μm and 25μm spheres. Further comparison of micro-

sphere concentration in the arterial blood and nutrient venous blood from the dog tibia indicated that the 15μm diameter spheres did not shunt through the bone significantly. However the use of the nutrient vein as a venous source of blood was questioned by Cofield et al (1975) who have stated that this vein drains only 10% of the tibial vascular bed. If this is the case then the findings of Gross et al must underestimate the percentage of microspheres non-entrapped when the venous sampling method is employed.

The problem of non entrapment has also been investigated by Tothill et al (1987) who examined the concentration of microspheres in the femoral venous blood after the spheres were injected into the tibial nutrient arteries of dogs. In this study the entrapment of only 15 ± 5μm diameter spheres was investigated. They found little evidence of non-entrapment, only 1.7% ± 2.6% (mean ± SD for 10 perfusions) of the administered activity found in the venous blood. However these authors suggest that dilation of vessels or opening of non-nutrient arteriovenous bypasses can lead to shunting of microspheres.

Thus, arterio-venous shunting does occur but the limited evidence suggests that 15μm diameter spheres are shunted only to a minimum extent. In general this size of microsphere is mostly trapped in bone but adverse conditions such as dilation of the vessels can lead to shunting and non-entrapment. Since the size of the rat prevents this type of study it has been assumed that from this evidence the 15μm diameter microspheres are best suited for the measurement of bone blood flow, the percentage of non-entrapment low and thus not an important factor in the estimation of flow. This was confirmed to some extent by analysing the lung of one animal, the percentage of activity in this tissue representing a very small proportion of the actual dose (less than 2%). However if all these microspheres pass through bone, this would be a very high proportion of the bone microspheres.

3.6.2. Control values for bone blood flow and mineral clearance

Assuming that the choice of cannulation site, microsphere size and number adequately fulfils the criteria for the use this method to measure bone blood flow the next topic to be considered is the control values of flow and clearance and how these compare with the findings of other authors.

1. Bone blood flow

These values fall into two groups for flow.

Table 3.12. Control blood flow, mean ± standard deviation

Combined flow for infusion or injected animals

Flow 1 0.36 ± 0.184 (SD) Flow 2 0.30 ± 0.114 (SD) n=20

Control group 3

Flow 1 0.32 ± 0.124 (SD) Flow 2 0.33 ± 0.125 (SD) n=21

Range of all measurements 0.087 - 0.698

Both these groups have mean flow values that are greater than the findings of other authors. However the range of measurements does include those of other investigators e.g. Fredrickson et al (1955) bone blood flow at least 0.1 - 0.3 ml/min/g of tissue. The study of Hruza et al (1969) gives a control value for tibia blood flow, of a ten week rat weighing 250g of 0.14ml/min/g, half that of the mean values presented in this work. However bone blood flow was measured using the bone clearance technique of Copp and Shim (1965) and as pointed out in the introduction this method assumes complete extraction (which is not the case) and thus this value can be questioned. In this respect the value is most likely to be an underestimation.

Schoutens et al (1979) compared the measurement of bone blood flow using strontium-85 microspheres and clearance of calcium-45. Although the results are in the form of plasma flow in bone ml/min/100g by using a mean figure for the haematocrit (46%) these can be converted in to flow in bone per gramme of tissue. That is multiply the plasma flow by 1.85 [1 ÷ (1-0.46)]

Table 3.13. The plasma flow values from Schoutens (1979) converted to bone blood flow

	Plasma flow ml/min/g	Bone blood flow ml/min/g
Femur	0.058 to 0.135	0.107 to 0.25
Tibia	0.051 to 0.114	0.094 to 0.21

These values are similar to that of the Hruza study but the estimates of flow using 45-Calcium are lower, in the femur clearance is 0.061 to 0.103 ml/mim/g and in the tibia this is 0.056 to 0.104 ml/min/g. As a result these authors suggest that bone clearance measurements are inadequate to monitor bone blood flow. If this is the case either the measurements of flow in this study are low or the method used by Hruza is more efficient as both papers give similar ranges of measurements for flow using the microsphere and clearance methods.

This however does not account for the higher blood flow estimates found in the present study. In the study of Schoutens the microspheres are injected directly into the left ventricle and blood samples withdrawn from the tail at a constant rate (figure not given in paper), thus the main difference between this study and the work of Schoutens is the weight and age of the animals. This however should not give rise to these differences in flow in fact Hruza and Wachtlova (1969) found that blood flow in bone dropped with increasing age (accompanied by an increase in weight). Similarly MacPherson and Tothill (1978) found a significant negative correlation for the blood flow of the tibia-fibula, jaw and femur. They did not however find a significant change in skeletal blood flow in the conscious rat with an increase in age or weight.

It is difficult to say why the results of this study are higher than those of the above authors. Possibly the age of the animals was of importance, some animals in the others studies may be immature thus having a slightly different blood supply because fusion of the metaphysis has not yet occurred however this is unlikely. The quantity and method of anaesthesia could have a part to play in these findings, Schoutens uses pentobarbital, 12mg ip. for a 240-260g animal, roughly 5mg per 100g. In this study the concentration

per 100g was 6mg ip. The animals are sensitive to the levels of anaesthesia and possibly the higher flow values reflects the effect of the anaesthetic.

A plot of all the control blood flows i.e. the cobalt flow of all the 'bone blood flow animals' shows that the blood flows are relatively normally distributed (Figure 3.10.). The mean of these 93 values is 0.33 with a standard deviation of 0.11. The distribution is slightly skewed (0.361) but this is not to a large degree so it is acceptable to say that the flow measurements of this study are normally distributed with a small plateau around the mean (indicated by a negative kurtosis, -0.15).

2. Strontium clearance

There are also two control groups in which strontium clearance has been estimated.

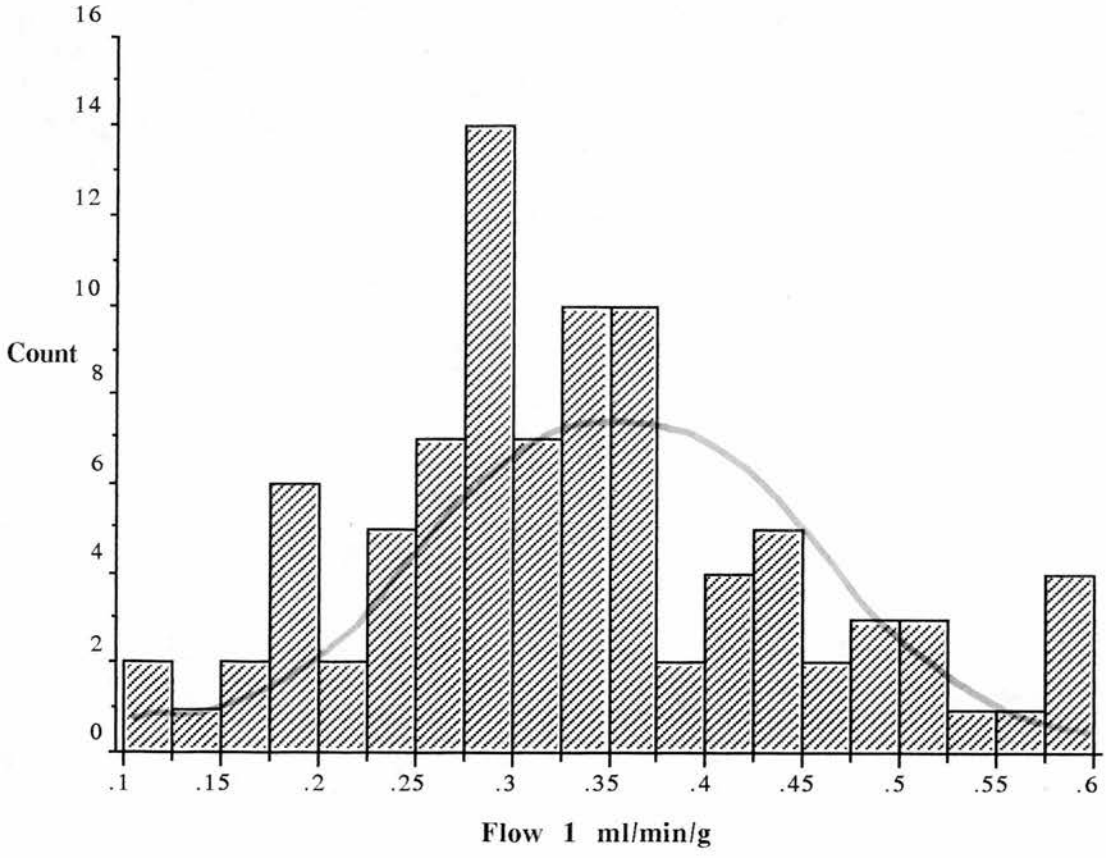
Control group 2	Sr clearance	0.46 ± 0.18	n=20
Control group 3	Sr clearance	0.22 ± 0.072	n=21

These two groups are significantly different and so are treated as two separate mean values in this discussion unlike the mean flow values which are similar enough to be grouped into one range.

Control group 2 represents animals where in all but one strontium extraction is greater than one. Strontium extraction is calculated from clearance and flow, thus a value of greater than one means that clearance is greater than flow, as clearance is dependant on flow this should not be possible in any animal. The possible reasons for this include over estimation of clearance through post mortem migration of strontium or because of streaming of microspheres, leading to under estimation of flow.

The post mortem migration of strontium has been investigated by by Tothill and MacPherson (1978). These authors found an approximately linear uptake of of bone radioactivity in the post mortem animal with time. The proportion of this increase depended upon the initial concentration of the bone-seeking nuclide, and ranged from a factor of 1.5 to 4.5 over a one hour period. Thus the effect of post mortem migration of strontium-85 from the soft tissue to bone can affect the accuracy of uptake measurements if there is any delay in removal of the bones. This post mortem uptake has been

Figure 3.10 Histogram of all the flow 1 values for the experimental rats with the normal distribution curve superimposed (n = 93)



confirmed by Dacke and Shaw (1987) who compared the uptake of strontium in non-microwaved and microwaved groups, finding it to occur in the untreated group.

In this study post mortem migration is negligible as the bones are removed and cleaned of all soft tissue immediately after sacrifice so this cannot be a factor in the calculation of strontium clearance. Thus the next consideration is the underestimation of flow possible through streaming of microspheres. This is discussed in the section investigating bone and caudal haematocrit.

Before looking at the possibility of streaming the values for control group three will be considered. In this case the strontium extraction values are all less than one, the range is 0.48 to 0.943 with a mean of 0.68 ± 0.11 (SD). Thus the randomness of animal choice or the shortening of the procedure time because of familiarity with the technique has removed the problem of high clearance values. The actual values for clearance are higher than those of Schoutens et al (1979). However the standard deviation of 0.072 is small indicating little variation occurring but, the small numbers make it difficult to compare this group with the of distribution of a normal population.

Thus although the values in this study are higher than those of other authors the results of flow and clearance are of an acceptable level with minimal variation to allow a comparison of drug effect on these variables to be made.

3.6.3. Caudal and bone haematocrit

Although the experimental control group do not have clearance values greater than flow this does not explain why such a problem occurred consistently in the original control group. In an attempt to explain this problem the haematocrit of bone and caudal arteries were examined.

Brookes (1965) and Tøndevold et al (1982) found that in long bone there are regional differences in plasma and erythrocyte volumes. This gives rise to associated regional differences in tissue haematocrit. Neither however have assessed the whole bone, thus making comparison of these results and those presented here difficult. Apart from the

femoral neck the values estimated in Tøndevolds' paper are all less than those presented in Table 3.6. Tøndevold has stated that although the haematocrit in bone depends on the location of measurement it is in the range of 50-75% of the arterial haematocrit. Here the values range from 47 to 100% with the two values obtained using the 111-In label greater than the 75% value. Although the paper of Tøndevold and Erickson involves work in dogs, assuming an arterial haematocrit of 40% this alone does not account for the degree of variation between this and the results of my study.

Brookes also found the haematocrit of bone to be proportionally less than the arterial haematocrit. The reason for this was outlined by Fahraeus in 1929 who found that the blood streaming in narrow tubes had a higher plasma content and lower corpuscle content than that streaming in larger tubes. He also found that the layer of fluid next to the vessel wall is mainly stationary and consists largely of the plasma fraction while the axial fast flowing stream contains a large percentage of the corpuscle fraction. Thus with decreasing tube diameter there is an associated decrease in haematocrit. These findings account for the decreased haematocrit in bone found in most of the animals in this study but does not shed any light on the difference in the estimates when different labels are used. Some factors which could give rise to this are discussed in the following pages.

1. Albumin egress

Tøndevold (1983) suggests that the egress of albumin occurs in the first few minutes the concentration leveling off to that of other markers 5-6 minutes. This would invalidate the use of albumin as a plasma marker. In this study the withdrawal of blood commenced within three minutes of marker injection, ceasing after a further two minutes. Thus the maximum time for albumin to egress is five minutes. The same method is used in all six animals therefore any over-estimation of plasma volume should be consistent and this egress of albumin is unlikely to be the cause of the different plasma erythrocyte volumes obtained.

2.Plasma labelling with red blood cell label

An over estimation of erythrocyte volume (99-Tc) could have been caused by the labelling of the plasma with the red cell label. The % of label in the supernatant indicating to what extent this occurred. When analysed the supernatant was found to contain less than 1% of any of the labels when compared to the content of the corpuscle fraction. Thus this was not an important consideration and insufficient to cause high haematocrit volumes.

3.Blood sample volume

A small blood sample may provide spurious counts resulting in invalid volumes. All the samples collected were in the range 3-4.5ml, and sufficient to provide acceptable counts. The blood samples were therefore not a problem in the calculations.

4. Assumed haematocrit

As stated in the results the haematocrit used in the calculations was assumed to be 45% but in reality this varied from animal to animal. The blood samples used to estimate the tail haematocrit gave similar values of total volume 0.97 ± 8E-3, as expected since 1ml was used each time, but varied in their red cell content from 0.38 - 0.57. Thus accounting for the variation in the measured tail haematocrit. This variation in the arterial haematocrit therefore affects the calculated tissue haematocrit. Using the tail values to calculate the bone haematocrit the situation changes. Firstly there are only two animals in each group, and secondly the mean haematocrit is now in the range 32-38 instead of 24-41 but with an increase in two of the standard deviations .

i.e. 99-Tc	34.5 ± 9
111-In	38 ± 3
51-Cr	32.5 ± 5

This suggests that the assumed haematocrit of 45% played an important part in the variation and the significance if the difference between the results when different labels

are used. This statistical significance is now lost. However this does not change the % value with reference to the arterial haematocrit.

Using these values the mean for the three labels is 35 ± 4. This value represents only 71% of the arterial mean which is within the limits suggested by Tøndevold. Therefore the use of the caudal artery as a reference organ in the estimation of bone blood flow appears to require a correction factor. However before stating that such a factor should be incorporated into the calculations note should be taken of how inconclusive the results are: the extent of the plasma volume variation and the variation in the measured arterial haematocrit the main problems.

The use of albumin as a plasma indicator is questionable. Its ability to pass to the extravascular space has been noted by many authors, but the extent of this over estimation when using this indicator is unknown. Thus measurement of plasma volume can only be an approximation and difficult to reproduce since plasma volume is capable of rapid fluctuations.

Measurement of red blood cell volume, on the other hand, is usually accurate and gives reproducible results. Here when 51-Cr and 111-In have been used, in one animal the measured volume is low. Since the number of animals used is only two this could have lead to a lowering of the mean erythrocyte volume. Only an increase in the number of animals used would allow this to be confirmed. Thus here animal number not method creates a difficulty in interpretation of the data.

The use of an assumed arterial haematocrit, such as 45% (Brookes) incorporates a degree of variation into the calculation. Removal of this by using the measured arterial haematocrit limits the extent of this variation therefore highlighting the difficulty of calculating a correction factor which could incorporate this. Here the arterial haematocrit ranges from 40-59% with the arterial percentage ranging from 47-100%. The mean value for all six animals allows a correction factor of 30% to be calculated. However this would cause a variation, the degree of which is represented in the variation in the arterial percentage.

Thus although this work points to a difference in the haematocrit of bone and caudal artery the actual degree of this difference is not conclusive. The study would have to be repeated incorporating other plasma labels i.e.125-I fibrinogen and increasing the number of animals. The suggested cause of the different haematocrits is that bone is richer in plasma and poorer in corpuscles than the caudal blood supply. To what extent this occurs I am not certain but this could give rise to an over estimation of strontium and an under estimate of the microsphere counts. This could account for the strontium extraction values that are greater than one in control group two.

3.6.4. Pump setting

Withdrawal of blood at a constant rate is a requirement of this procedure. Unfortunately the actual withdrawal rate is not cited in the methods of any of the comparable papers making it impossible to draw any comparison between these and the present study (Schoutens et al 1979, Tothill and MacPherson 1980). However this study suggests that the 0.197ml/min rate is the most suitable withdrawal setting for the rat producing the most reproducible results. At higher rates the vessel tends to clamp down restricting the collection.

The actual withdrawal rate varies around this setting. In some cases blood collection is sluggish and the rate is less than this while in other animal the flow is so great that the collection rate is above this value. Thus to minimise this variation the actual flow rate is estimated by weighing the syringes before and after collection then dividing the weight of the sample by the specific gravity of blood. The per minute rate is then estimated by dividing this weight by the time of collection i.e. 2 minutes or 4.5 minutes. The withdrawal timing is not begun until the blood appears in the syringe to ensure that the time period represents actual withdrawal of blood. This value is then used as the pump rate in the calculation of bone blood flow.

3.6.5. Administration of drug and experimental variation

The chosen method of administration depends on the half life of the drug under investigation. This study has confirmed that neither method has any direct effect on the animal (3.5.4.).

The degree of experimental variation is important in determining the reproducibility of the results. As stated in 3.5.4. the high percentage of variation in the combined control group is unacceptable. Randomness of selection of animals, drug choice and dose minimise this experimental variation to an acceptable level. Therefore this method of selection of animal and drug has been incorporated in the protocol of bone blood flow measurement and the animals were ordered in large sibling groups i.e. 16-24 animals in one group.

3.7. Conclusions

These are presented in the form of the selected protocol. Further details of the specific methods are included in the approprite chapters i.e. chapter 4 vasoactive agents.

PROTOCOL

1. Dissect and cannulate the carotid and caudal arteries (3.2.2.).
2. Connect animal to infusion pump via the carotid cannula - 6ml/hour.
3. Inject 57-Cobalt microspheres via the carotid cannula.

Begin withdrawal after thirty seconds (0.197ml/min), ceasing after ninety seconds.

4. Inject or infuse the test solution (Random choice of dose and drug).

The time period between administration of drug and the withdrawal begining depends on the agent. Begin withdrawl (0.197ml/min) and thirty seconds later inject Tin-113 and Sr-85. Cease withdrawal after a total of 4.5 minutes.

5. Scarifice animal with a saturated KCl solution via the tail cannula and immediately remove tibiae and femora and a sample of the upper quadriceps.

Weigh all samples, including blood samples and analyse for radioactivity.

A correction factor for the difference between caudal and bone haematocrit cannot be included in the calculation of bone blood flow but this is a possible area of further investigation.

VASOACTIVE AGENTS

4.1. Introduction

Vasoactive agents are known to affect blood flow through action on blood vessels. This takes two forms: 1. vasoconstriction and 2.vasodilatation.With respect to these the action a variety of agents have been investigated, these include noradrenaline, adenosine, isopropaline and adrenaline.

Noradrenaline is a neurotransmitter of the sympathetic nervous system, through stimulation of the alpha-adrenergic receptors it produces vasoconstriction. Gross et al (1979) found that noradrenaline had a direct constrictor effect on bone and marrow, increasing arterial pressure and decreasing bone blood flow. These authors also examined the vasodilator responses in bone blood vessels to the effects of adenosine. Both arterial pressure and vascular resistance was decreased with adenosine treatment. It is suggested that these act through a direct effect on the vascular smooth muscle.In bone vessels the response is of a similar magnitude to that of skeletal muscle. In addition to investigating the effect of noradrenaline and ATP on the exchange processes in bone McCarthy and Hughes (1985) also examined bone blood flow and arterial blood pressure. During infusion of noradrenaline flow decreased significantly while vascular resistance increased. The reverse occurred during ATP infusion. This supports the vasoconstrictor effect of noradrenaline and suggests that ATP acts as a vasodilator thereby allowing blood flow to increase and reducing vascular resistance. Driessens and Vanhoutte (1981) also assessed the responsiveness of bone to vasoactive agents. By measuring the perfusion pressure of an isolated tibiae following administration of the agents they confirmed that noradrenaline caused constriction of bone blood vessels.

4.2. Choice of Drug and dosage

The effects of noradrenaline and adenosine in terms of vascular response has been well detailed. However few of these studies have related this action to a change in blood flow or clearance of minerals in bone. This knowledge of the vascular action of these agents allowed some prediction of their effect on bone blood flow to be made in advance. Thus the work of previous workers provided a good foundation for this choice and ensured that drugs with opposite vascular effects were compared.

The choice of dosage was made on the bases of earlier work carried out in this department (Davies 1986). Here noradrenaline at doses of 0.57 and 1.24µg/Kg/minute (over 5 minutes) were found to significantly reduce blood flow and increase arterial blood pressure. In a 350g rat these doses were equivalent to 1 and 2µg of noradrenaline per animal. In order to plot a dose response curve an intermediate dose of 1.5µg per animal was also chosen. Thus the selected noradrenaline doses were 0.2, 0.3 and 0.4µg /minute. These represented pharmacologically active levels: in humans about 2-4µg per minute of norepinephrine bitartate is used in treatment of hypotension (Goodman and Gilman 1980). Thus, assuming an average weight of 70Kg for a human, the dose administered to a rat represents about 40-80µg in a human per minute over a five minute period. The choice of infusion of this drug was due to its rapid inactivation by the same enzymes that methylate and oxidatively deaminate epinephrine. Test injections of 2µg bolus noradrenaline (equivalent to 0.4µg/minute) had no apparent effect on either blood flow or blood pressure suggesting that the agent is quickly inactivated. However infusion of the same dose resulted in an effect on arterial pressure after about thirty seconds which remained until the end of the experiment. Thus it was decided to infuse the solutions and wait 30 seconds before begining withdrawal to allow the drug to have an effect. The above study also included ATP. At the highest dose i.e. 0.724 mg/Kg/ minute an increase in blood flow and a decrease in blood pressure was observed. This dose represents 1.25mg/350g animal infused over a total of five minutes. Other doses

in this study were equivalent to 0.25, 0.45 and 0.80 mg/ animal. The 0.25mg dose was then selected as the lowest dose and 0.75mg selected as an intermediate dose to complete the dose curve. Since the effects of ATP has not been as extensively studied the literature does not appear to cite basal levels of this agent thus it was difficult to find out if these represented pharmacological or physiological levels. In order to compare the two agents ATP was made in similar solution to noradrenaline and infused at the same rate.

4.3. Preparation of injected dose

Both noradrenaline and ATP were bought in powdered form; the required concentration was achieved by weighing out some of this and dissolving it in buffer. In each case 10ml of the solution was made up at any one time, this ensured that the 25ml syringe contained a large enough volume to allow the infusion pump to function correctly. Thus on occasions the same batch of solution was used in more than one animal.

a) Noradrenaline (Sigma - 1g.) -- 1,1.5 and 2µg per rat, 0 C°< .

These dose are equivalent to 5,7.5 and 10µM. infused over five minutes.

Over the infusion period 0.5ml was infused. Thus for a 10ml solution of the correct concentration 20, 30 and 40µg of noradrenaline was required. The balance however could not weigh such small quantities so a concentrated solution was made up using 2mg dissolved in about 1ml saline and made up to 10ml using buffer. For the 1µg dose 0.1ml of this concentrated solution was then made up to 10ml using buffer (0.15 for the 1.5µg and 0.2 for the 2µg doses).

b) ATP (Sigma; 500mg Vanadium free from Equine muscle)

-- 0.25,0.75 and 1.25mg per rat, 0 C°<.

These doses are equivalent to 0.32, 0.96 and 1.6mM. infused over five minutes. ATP was also made up in 10 ml quantities, 5,15 and 25mg were required for each respective dose.

These were then dissolved in small quantities of buffer before being made up to a 10ml volume.

In both cases it was important to ensure that the powder was completely dissolved especially at the higher doses, efficient mixing in the 10ml volume was usually sufficient. The solutions were made up when required and the choice of dose randomised to minimise variation between dose groups. Thus in any one sibling group a variety of doses or drugs were used.

4.4. Experimental procedure

a) Noradrenaline and ATP (Figure 4.1)

The prepared animal (see 3.2.2.) was connected via the carotid cannula to an infusion pump and the pressure recorder. This was achieved using two three-way taps, to the first a Braun infusion pump was connected and to the second a pressure transducer. Lost blood was replaced by the pump which infused buffer or the test solution at a constant rate (6 ml/hour). This infusion also ensured that the carotid cannula remained patent. Needles were pushed under the skin of the animal at both fore paws and one hind paw in order to obtain an electro cardiograph (ECG) and average heart rate. The blood pressure of the animal was allowed to stabilise, and then the tail cannula was connected to a withdrawal pump set at 0.197ml/minute, the syringes used for collection had been previously weighed so that the of actual collection rate could be calculated.

1. Control blood flow.

Buffer was infused over the control flow period. Blood collection commenced and thirty seconds later Co-57 microspheres were injected into the heart via the first tap. Blood was withdrawn for a further ninety seconds.

2. Test blood flow.

After ensuring that the pressure was stable the test run was begun. The syringe containing buffer was replaced with a syringe containing the test drug, blood was

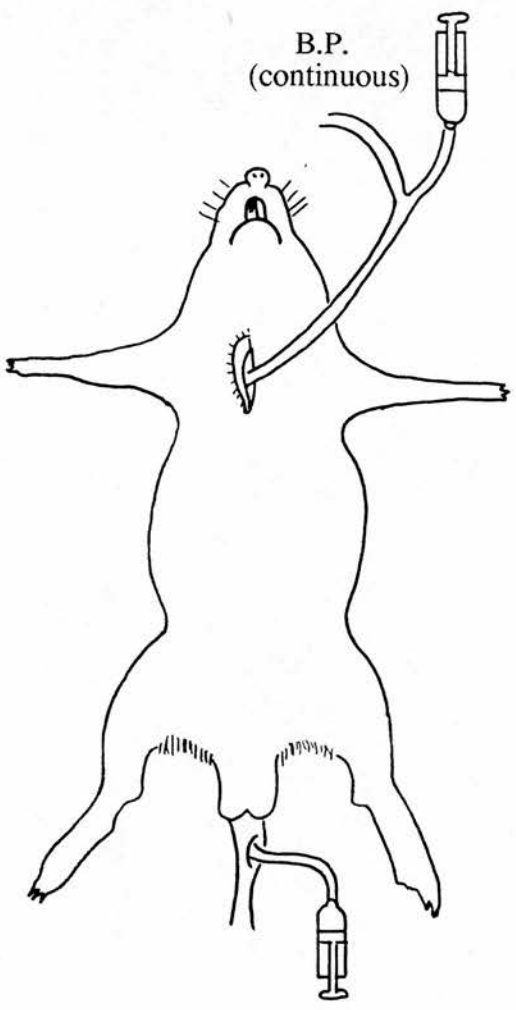
allowed to come back into the carotid cannula and the syringe and cannula then reconnected. Thus the start of infusion could be visualised through clearing of the cannula. After thirty seconds blood withdrawal began into a second syringe. At one minute Tin-113 and Strontium-85 were injected. Blood withdrawal ceased after 4.5 minutes.

At the end of the experiments the animals were sacrificed using KCl injected via the tail cannula. Both tibiae and femora were removed quickly to minimise post-mortem migration of ions to the bone. A muscle sample from the upper quadriceps was also taken. The bone, muscle and blood samples were then weighed before counting.

b) Control animals

In these animals the same method was followed but during the test run a solution equivalent to the test solution minus the drug was infused i.e. Krebs Ringer buffer.

Figure 4.1. Schematic Presentation of Procedure



Infusion pump

To carotid artery:

1. Co-57 µspheres.
2. Drug (per animal
   a. INFUSION
   ATP 0, 0.15, 0.75 and 1.5mg
   Noradrenaline 0, 1.0, 1.5 and 2.0µg

Sn-113µspheres
Sr-85.

Withdrawal pump

From caudal artery:

1. 2 minutes collection
2. 4.5 minutes collection

4.5 Results

a). Noradrenaline

This group was composed of twenty six animals, in eight there were insufficient microspheres in either of the blood samples and two died during the procedure. The results of the remaining sixteen animals are shown in tables 4.1 - 4.4. These include details of blood flow in bone and muscle (Tables 4.1. and 4.4.), strontium clearance in rat bone (Table 4.1.), arterial blood pressure (Table 4.3.) and vascular resistance in bone and muscle (Tables 4.2. and 4.4.).

Noradrenaline treatment causes a dose dependent reduction in blood flow with a rise in arterial blood pressure, strontium extraction and vascular resistance (Figures 4.2.,4.4, 4.6. and 4.7.).

The change in blood flow is significant at the 1.5 and 2.0µg levels ($p<0.05$). A comparison of the mean Flow 2 with mean Flow 1 at each dose shows that the experimental flow is reduced compared with the control (Table 4.1.).

i.e. Dose % of Flow 1

1.0µg Flow 2 = 77%

1.5µg Flow 2 = 56%

2.0µg Flow 2 = 62%

The line of regression for this change in flow is significant and negative, this implies that it is the increasing noradrenaline concentration that is responsible for the degree of change. In fact the r squared value suggests that this is the cause of some 30% of this variation.

Accompanying this change in flow is a change in arterial blood pressure. Blood pressure significantly increases with dose (ie. the slope of the regression is significantly different from 0, Figure 4.4.). The arterial blood pressure is measured at four points which are detailed in table 4.3. The pressures at B1 (point of Co microsphere injection) and at B2 (the begining of the test blood flow) should be relatively similar, and in all but the 1.0µg

dose this is the case. But even the difference at this dose is not significant. However B2 is used as the arterial pressure level in the calculation of a change in pressure in case the use of cobalt microspheres has in some way affected the pressure (although this does not appear to be the case). The arterial pressure at the point of tin microsphere injection is represented by the B3 measurement while the point of maximum drug effect is represented by B4. Although none of the B3 or B4 measurements are significantly different within each dose group the differences in the values are important in the calculation of arterial pressure change. Therefore in each case B4 is used in this calculation and pressure change is calculated as follows; B4 - B2.

The arterial pressure rises almost immediately after infusion has begun and although the degree of change is similar for each dose the length of this effect is subject to the concentration of noradrenaline (Figure 4.5). Thus at 1.0µg the pressure returns to the original level by five minutes while at 2.0µg pressure is still at a high at three minutes and is only beginning to decline at five minutes.

Vascular resistance displays the same pattern as blood pressure i.e. there is an increase with increasing concentration, the 2.0µg tin value is 220% of the cobalt value (Table 4.2.). This effect could be expected from the decrease in flow ie. a vasoconstrictory response reducing the flow through increased vascular resistance. Once again the response is caused by the concentration of noradrenaline, indicated by the regression value, r squared being 0.35 (Figure 4.7).

Strontium clearance is unaffected by noradrenaline treatment (Figure 4.3.). At no dose is there any significant difference from the control value (Table 4.1.). On the other hand strontium extraction increases significantly with a significance difference from the control group found at the 1.5 and 2.0µg doses (Table 4.2.). Extraction is dependent on both flow and clearance thus if clearance is relatively constant, as is the case here, it is the change in flow that gives rise to the effect on extraction. Comparison of the slopes of figures 2 and 3 confirms that the lines of regression are significantly different ($t=2.12$, $d.f.=28$ $p<0.05$) supporting the view that the change in flow that gives rise to the change in extraction.

Neither muscle flow or vascular resistance appear to be significantly affected by noradrenaline. The standard deviations for vascular resistance are high illustrating the range of the individual values (Table 4.4.).

Noradrenaline therefore causes a significant reduction in bone blood flow but has no effect on strontium clearance. Thus the drug does not alter clearance independantly of it s action on flow. The two variables have been plotted against one another in figure 4.8. Comparision of this figure with the control animal plot (figure 3.9) shows that the two are similar statistically ie. t=1.35, D.F. = 26. These results indicate that there is some relationship between flow and clearance however a significant decrease in flow need not give rise to a similar significant decrease in clearance.

Finally flow 2 is plotted against blood pressure in the noradrenaline treated animals (figure 4.9). As the correlation is low (0.33) this suggests that the relationship between the two is weak.

Table 4.1. Effect of noradrenaline on blood flow, strontium clearance and blood pressure in rat bone

		Noradrenaline µg/animal		
	Control	1.0	1.5	2.0
Flow				
Cobalt	0.34 ± 0.13	0.35 ± 0.19	0.34 ± 0.11	0.32 ± 0.03
Tin	0.36 + 0.12	0.27 ± 0.07	0.19 ± 0.04	0.20 ± 0.07
@Sn - Co	0.02 ± 0.04	-0.08 ± 0.17	-0.15 ± 0.09	-0.13 ± 0.04
*** Sr clearance**				
	0.24 ± 0.09	0.20 ± 0.03	0.17 ± 0.05	0.19 ± 0.09
Blood Pressure Change				
(B4 - B2)	-2.50 ± 3	40 ± 26	39 ± 8	39 ± 13

@ Paired t-test --- 1.5µg $p \leq 0.05$; 2.0µg $p \leq 0.01$.

* Unpaired t-test (comparison with 0 dose) --- All N.S.

All values represent mean ± standard deviation.

Number of animals in each group (n) = 4.

Flow and clearance are measured in ml/min/g.

Blood pressure is measured in mmHg; at two points;

(B2) at the begining of the experimental run,

(B4) at point of maximum effect.

Sn = Tin. Co = Cobalt.

Table 4.2. Effect of noradrenaline on strontium extraction and vascular resistance in rat bone

		Noradrenaline µg/animal		
	Control	1.0	1.5	2.0
***Strontium Extraction**				
	0.66 ± 0.08	0.75 ± 0.18	0.90 ± 0.06	0.94 ± 0.21
Vascular Resistance				
Cobalt	425 ± 265	570 ± 554	498 ± 187	367± 176
Tin	390 ± 207	585 ± 250	1025 ± 331	809 ± 354
@Sn - Co	-36± 87	15 ± 496	528 ± 190	442 ± 208

@ Paired t-test --- 1.5µg $p \leq 0.02$; 2.0µg $p \leq 0.05$.

* Unpaired t-test (comparison with 0 dose) --- 1.5 µg $p \leq 0.01$; 2.0 µg $p \leq 0.05$.

All values represent mean ± standard deviation.

Number of animals in each group (n) = 4.

Vascular resistance is measured in mmHg/min/g(using B1 for calculating the cobalt value ad B3 the tin value).

Sn = Tin. Co = Cobalt.

Table 4.3. Mean and standard deviation of blood pressure measured at the four reference points in noradrenaline treated animals

			Noradrenaline µg/animal		
		Control	1.0	1.5	2.0
Blood pressure					
Cobalt	B1	119 ± 26	129 ± 29	155 ± 29	115 ± 46
Tin	B2	118 ± 26	140 ± 37	152 ± 86	114 ± 36
	B3	106 ± 30	146 ± 42	185 ± 35	144± 32
	B4	109 ± 27	151 ± 37	197 ± 29	142 ± 34

Number of animals in each group (n) = 4.

Blood pressure is measured in mmHg.

B1 and B3 Blood pressure at point of microsphere injection used for calculation of vascular resistance.

B2 Blood pressure at the begining of the experimental run, used for the calculation of blood pressure change (B4 - B2).

B4 blood pressure at the point of maximum drug effect.

Table 4.4. Effect of noradrenaline on blood flow, and vascular resistance in rat muscle

		Noradrenaline µg/animal		
	Control	1.0	1.5	2.0
Flow				
Cobalt	0.12 ± 0.03	0.12 ± 0.04	0.06 ± 0.04	0.08 ± 0.02
Tin	0.09 + 0.01	0.11 ± 0.05	0.12 ± 0.10	0.15 ± 0.05
@Sn - Co	-0.03 ± 0.02	-0.01 ± 0.07	-0.05 ± 0.08	0.07 ± 0.04
Vascular resistance				
Cobalt	1279 ± 614	1105 ± 282	1794 ± 897	1464 ± 552
Tin	1199 ± 362	1365 ± 348	3165 ± 3279	1017 ± 361
@Sn - Co	-80 ± 634	260 ± 476	1371 ± 3991	-446 ± 400

@ Paired t-test --- **Flow** 2.0µg $p \leq 0.05$.

All values represent mean ± standard deviation.

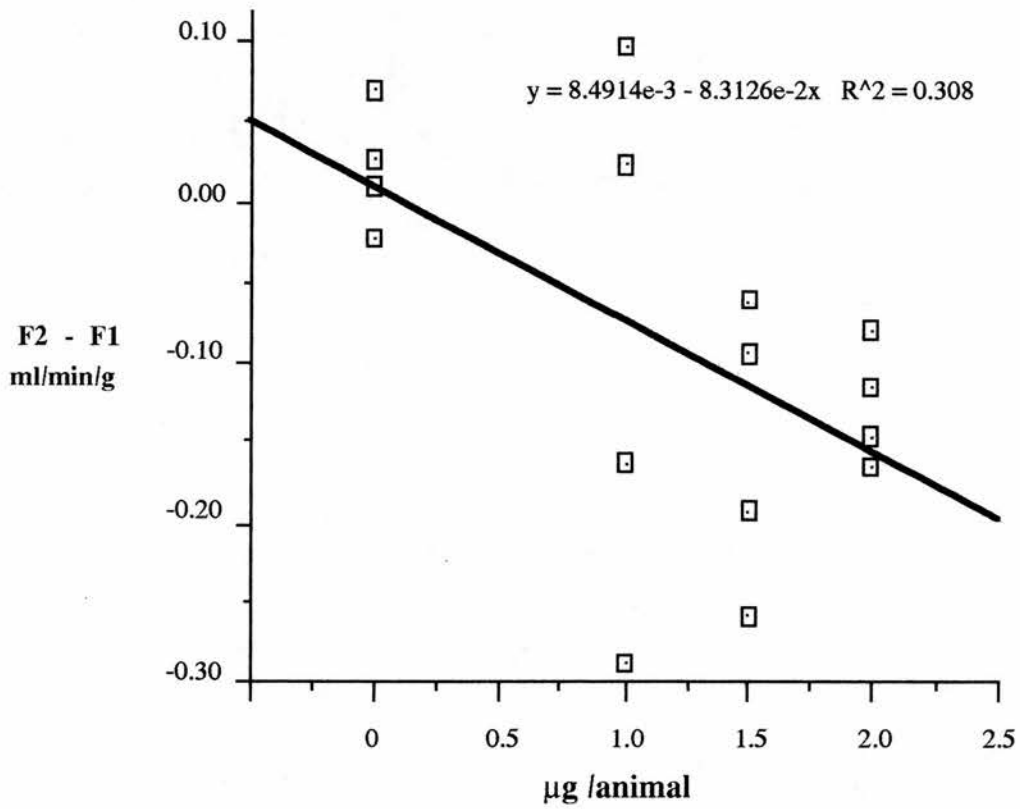
Number of animals in each control group (n) = 3.

Noradrenaline treated group (n) = 4.

Flow is measured in ml/min/g.

Vascular resistance is measured in mmHg/min/g (using B1 for calculation of cobalt value and B3 for calculation of tin value)

Figure 4.2. Effect of Noradrenaline on Bone Blood Flow

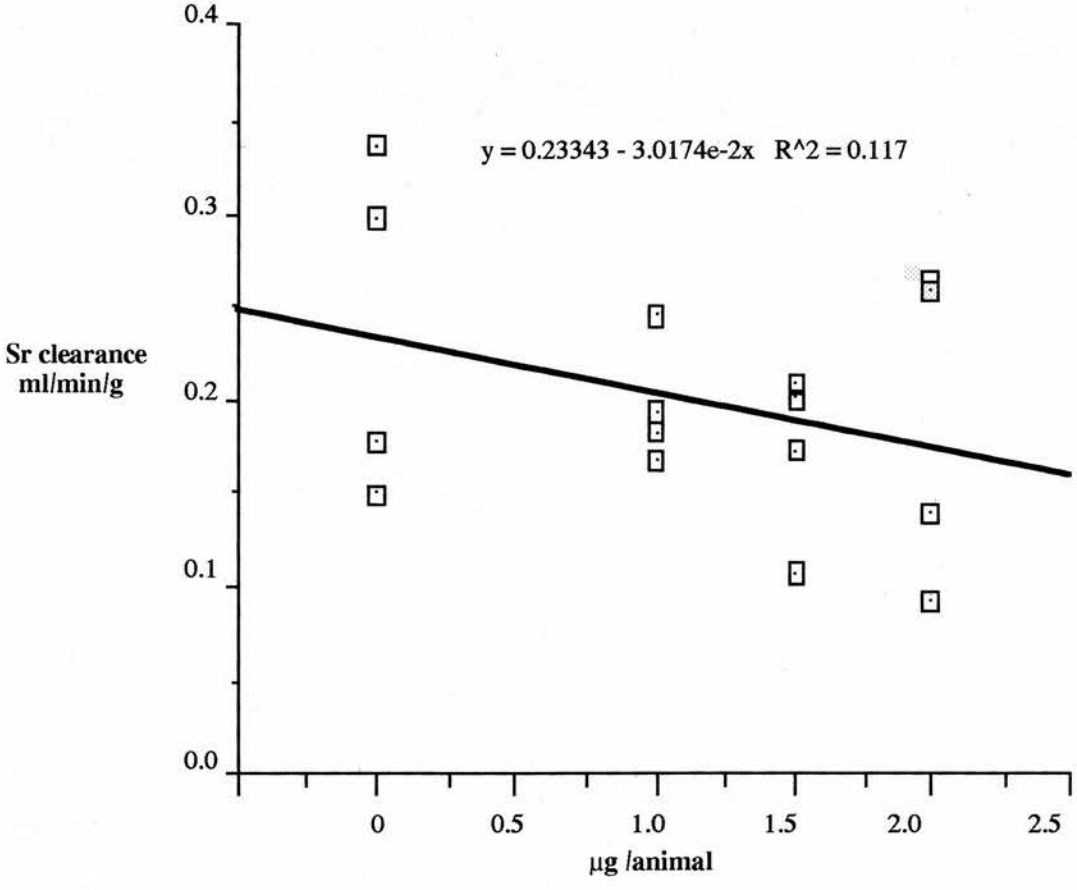


F1 = Control Blood Flow -- using Co microspheres.
F2 = Experimental Blood Flow --- using tin microspheres.

Significance of the regression , $p \leq 0.03$

Correlation coefficient (r) = - 0.55

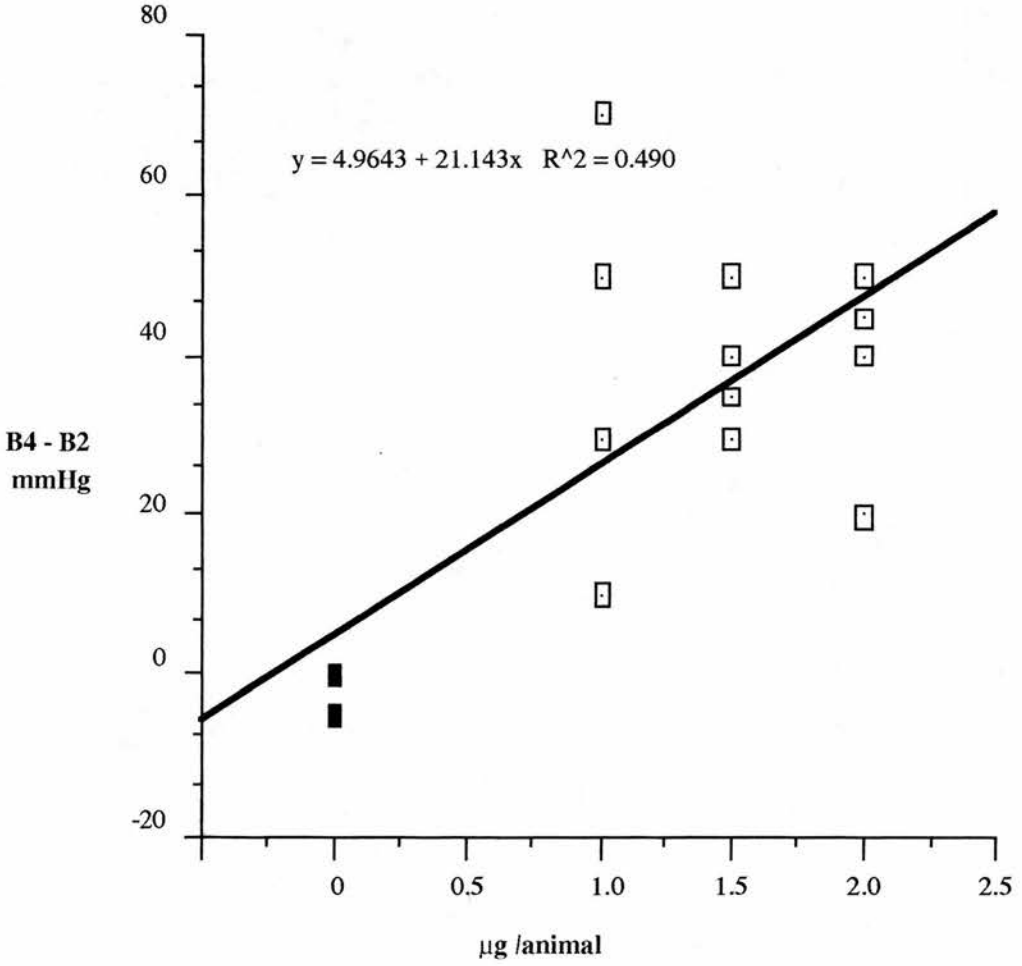
Figure 4.3. Effect of Noradrenaline on Strontium clearance



Significance of the regression, $p \leq 0.2$

Correlation coefficient (r) = -0.34

Figure 4.4. Effect of Noradrenaline on Arterial Blood Pressure



▮ Two points

B2 = Arterial Pressure measured at begining of experimental period .
B4 = Arterial Pressure measured at point of maximum drug effect.

Significance of regression, $p < 0.003$

Correlation coefficient (r) = 0.7

Figure 4.5. An example of the arterial pressure trace from an animal given 2.0μg Noradrenaline

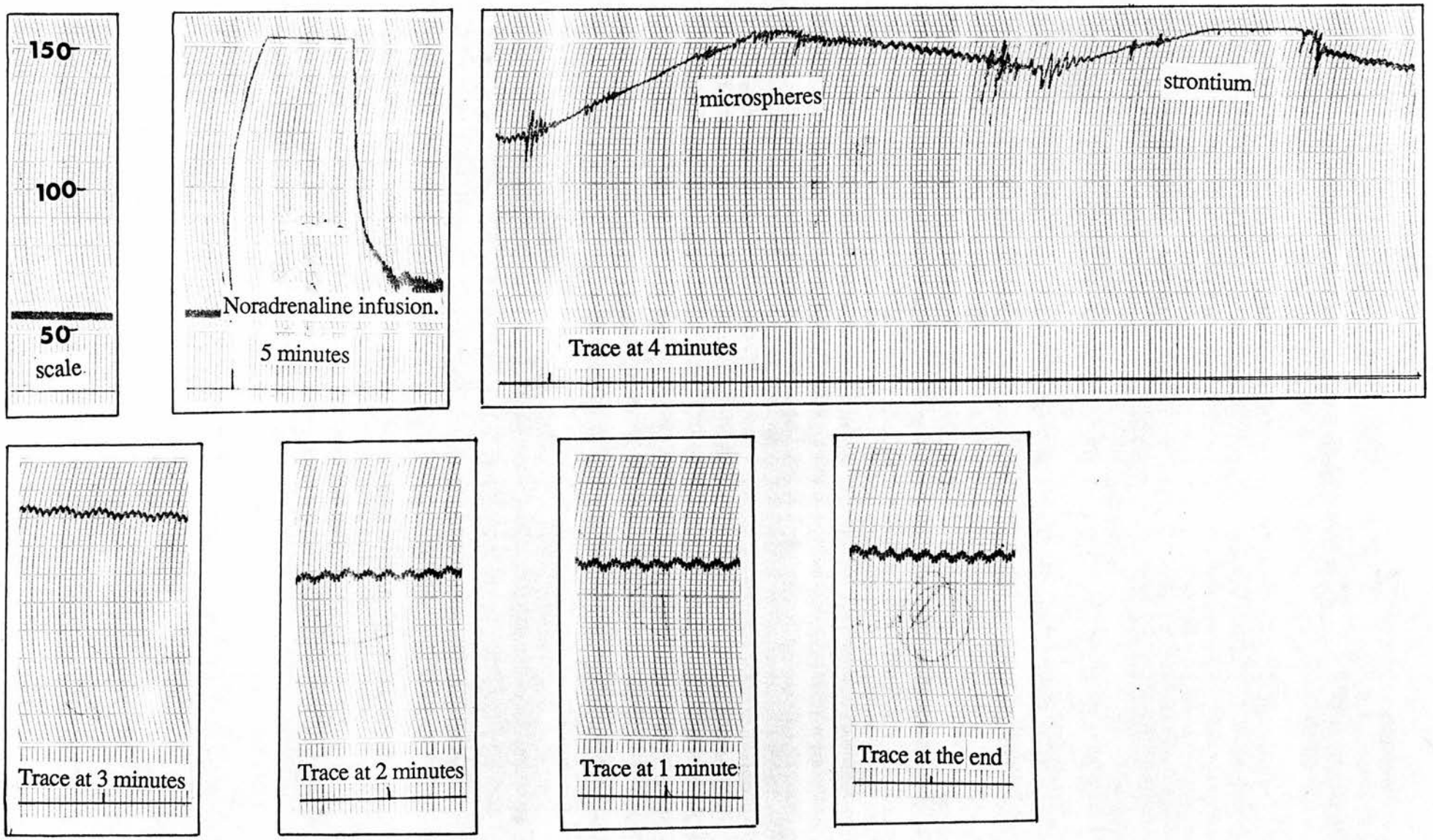
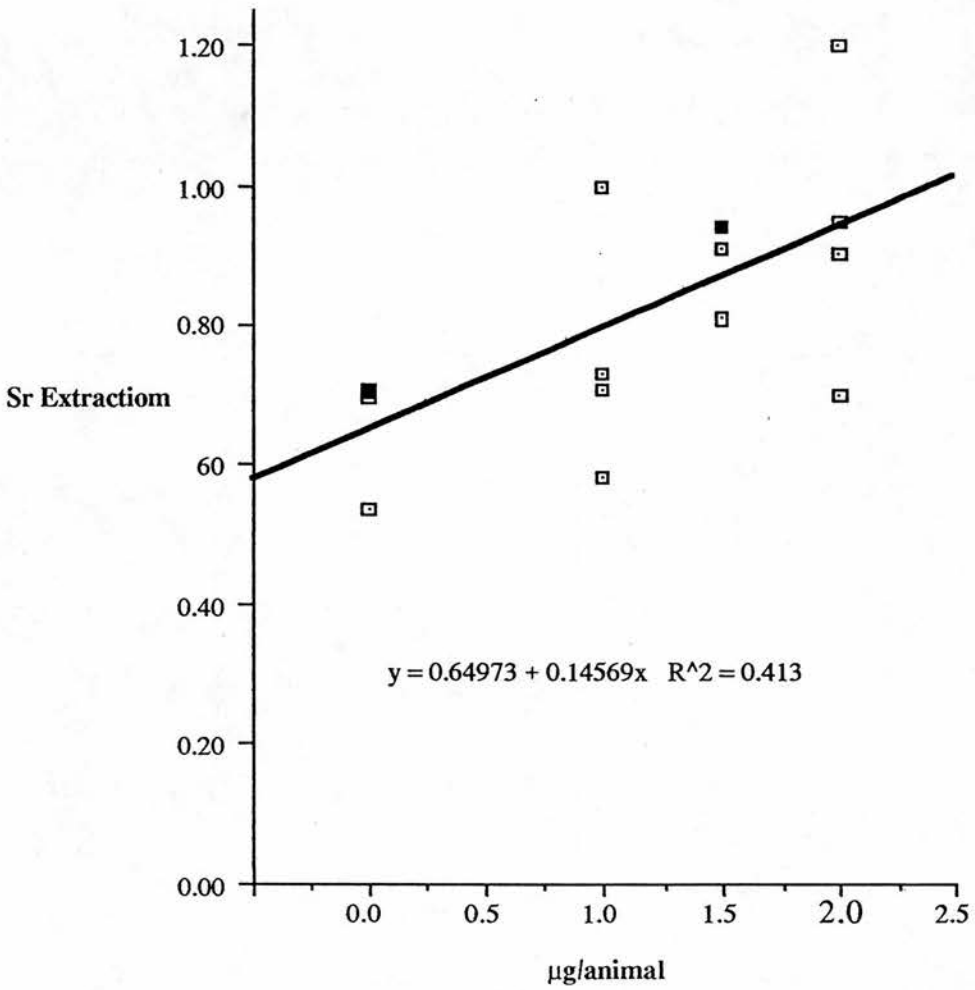


Figure 4.6. Effect of Noradrenaline on Strontium Extraction

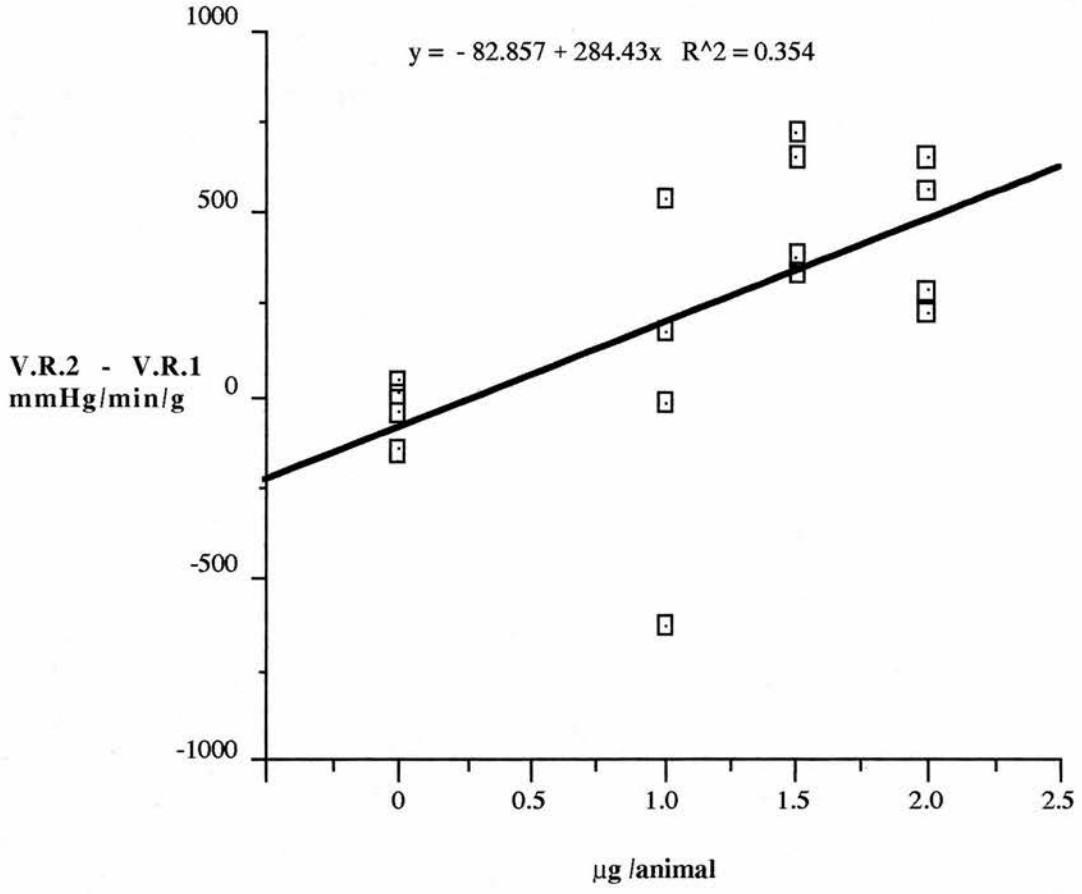


■ Two points

Significance of Regression, $p<0.007$

Correlation coefficient (r) = 0.64

Figure 4.7. Change in Bone Vascular Resistance with Noradrenaline Treatment

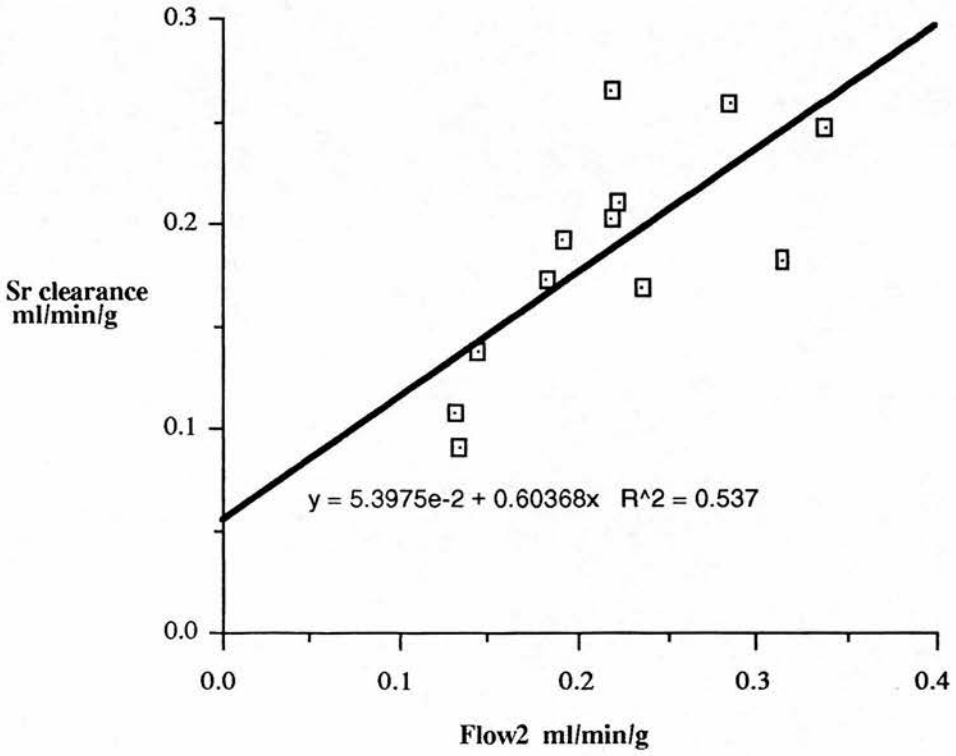


V.R.1 = Vascular Resistance as estimated from cobalt flow and blood pressure (B1.)
V.R.2 = Vascular Resistance as estimated from tin flow and blood pressure (B3).

Significance of the regression, $p \leq 0.015$

Correlation coefficient (r) = 0.59

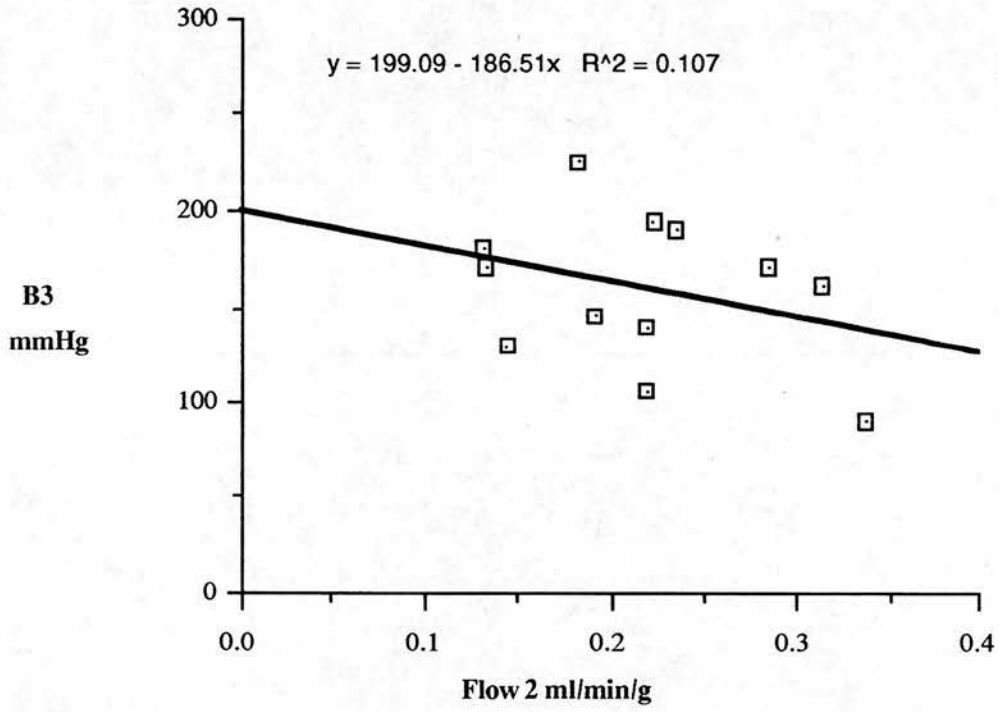
Figure 4.8. **Flow Versus Strontium Clearance in the Noradrenaline Treated Animals**



Significance of the regression $p \leq 0.033$

Correlation coefficient (r) = 0.73

Figure 4.9. Flow 2 Versus Blood Pressure in the Noradrenaline Treated Animals



Signifiacnce of the regression $p \leq 0.001$

Corelation coefficient (r) = - 0.33

b) ATP

In total twenty seven animals were treated with 0, 0.25, 0.75 and 1.25mg ATP. Of these two died during the procedure, in four there was a problem with collection of the cobalt blood samples, either an insufficient volume or the number of microspheres was inadequate; in another the collection of blood commenced after the addition of tin microspheres and finally in one animal their was insufficient ATP administered to meet the required dose. The effect of ATP treatment in the remaining nineteen animals on blood flow and vascular resistance in rat bone and muscle are dealt with in tables 4.5., 4.6. and 4.7. Table 4.5. also includes the drugs effect on clearance and the change in arterial pressure as a result of treatment. The actual values for arterial pressure are detailed in table 4.8.

In all but one area ATP had no significant action. Arterial blood pressure was significantly decreased by drug treatment, this decrease was dose dependent (Figure 4.12). The arterial pressure of the test period drops in relation to the initial pressure in each case (Table 4.8.)

i.e. B4 as a percentage of B2

0.25mg Pressure = 66%

0.75mg Pressure = 70%

1.25mg Pressure = 59%

Table 4.8. itemises the arterial pressure at the four points of interest. There is no significant difference between the B1 and B2 values indicating that the injection of cobalt microspheres did not have any effect on arterial pressure. The timing of the response to ATP is highlighted by this table. At thirty seconds (B3) the blood pressure is falling but at no dose has reached its lowest value. Reviewing the traces allows this timing of the maximum drop for each dose to be identified (Figure 4.13.). The maximum drop in pressure occurs after 1.5 minutes for the lowest dose (i.e. 0.25mg) and after two minutes for the other doses. By the end of the five minute period the pressure has returned to normal for all of the 0.25mg treated animals and for most of the 0.75mg

animals but is still low for the 1.25mg dose. Thus the length of the change in pressure is dependent upon the dose of ATP administered, the duration of effect increases with increasing dose.

Although ATP was selected because it was though it would increase blood flow this is not the case. Bone blood flow actually exhibits a negative trend, a decrease in flow with increased dose, but the line of regression is not significant (Figure 4.10.). At the 0.75mg dose this fall is over 50% of the initial level but only 25% at the 1.25mg dose (Table 4.5.). Although there are some differences in the mean values for strontium clearance this is not reflected in the regression line where r is virtually 0 and clearance remains constant (Figure 4.11.). As there is no change in clearance and little change in flow one would expect little change in strontium extraction. This is demonstrated in figure 14 the line of regression for extraction having a shallow slope (b=0.13) and is also not significantly different from zero. At no dose was any significant difference from the 0 group found when the mean extraction values were analysed using a students t-test (Table 4.6.).

Bone vascular resistance shows no change, the slope being shallow and the line of regression is not significantly different from zero (Figure 4.15.). Infact the values are widely spread with a large variation about the mean. Table 4.6 highlights this, the mean values for resistance change alternating between the negative and positive with large standard deviations.

Administration of ATP does effect muscle vascular resistance, however only at the 1.25 dose (Table 4.7). A plot of the line of regression for this is not significant. Similarly in no respect does muscle flow show a significant trend with respect to the regression line.

Figure 16 is a plot of flow versus strontium clearance in the drug treated animals only. This line is not significant different when compared to figure 3.9. (control animals) ($t = 0.46$, $D.F = 29$). Thus the ATP animals show the same relationship between flow and clearance demonstrated by the normal untreated animals.

Finally in figure 4.17 flow 2 is plotted against blood pressure, like the noradrenaline animals there is a low correlation value (0.55) indicating only a weak relationship.

Table 4.5. Effect of ATP infusion on blood flow, strontium clearance and blood pressure in rat bone

		ATP mg/animal		
	Control	0.25	0.75	1.25
Flow				
Cobalt	0.23 ± 0.09	0.27 ± 0.07	0.36 ± 0.15	0.40 ± 0.17
Tin	0.22 ± 0.09	0.24 ± 0.08	0.17 ± 0.06	0.30 + 0.06
@Sn - Co	-0.01 ± 0.03	-0.02 ± 0.11	-0.19 ± 0.10	-0.11 ± 0.14
*** Sr clearance**				
	0.16 ± 0.05	0.21 ± 0.07	0.12 ± 0.02	0.19 ± 0.04
Blood Pressure Change				
(B4 - B2)	0	-40 ± 34	-41 ± 23	-55 ± 16

@ Paired t-test --- 0.75mg $p \leq 0.05$

* Unpaired t-test (comparison with 0 dose) --- All N.S.

All values represent mean ± standard deviation.

Number of animals in each group : Control (n) = 4.

ATP (n) = 5.

Flow and clearance are measured in ml/min/g.

Blood pressure is measured in mmHg; at two points;

(B2) at the begining of the experimental run,

(B4) at point of maximum effect.

Sn = Tin. Co = Cobalt.

Table 4.6. Effect of ATP on strontium extraction and vascular resistance in rat bone

		ATP mg/animal		
	Control	0.25	0.75	1.25
***Strontium Extraction**				
	0.77 ± 0.12	0.89 ± 0.19	0.78 ± 0.41	0.66 ± 0.13
Vascular Resistance				
Cobalt	647 ± 315	483 ± 143	464 ± 257	434 ± 254
Tin	685 ± 186	482 ± 217	750 ± 354	354 ± 52
@Sn - Co	38 ± 136	-1 ± 210	285 ± 129	-80 ± 250

@ Paired t-test --- All N.S.

* Unpaired t-test (comparison with 0 dose) --- All N.S.

All values represent mean ± standard deviation.

Number of animals in control group (n) = 4.

Each ATP group (n) = 5.

Vascular resistance is measured in mmHg/min/g (using B1 for calculating the cobalt value and B3 the tin value).

Sn = Tin. Co = Cobalt.

Table 4.7. Effect of ATP infusion on blood flow, and vascular resistance in rat muscle

		ATP mg/animal		
	Control	0.25	0.75	1.25
Flow				
Cobalt	0.14 ± 0.12	0.09 ± 0.04	0.10 ± 0.04	0.14 ± 0.04
Tin	0.05 ± 0.03	0.13 ± 0.06	0.14 ± 0.16	0.16 ± 0.03
@1Sn - Co	-0.09 ± 0.13	0.04 ± 0.04	0.03 ± 0.15	0.02 ± 0.05
Vascular resistance				
Cobalt	3451 ± 5008	2307 ± 2441	1532 ± 687	942 ± 213
Tin	4211 ± 3700	923 ± 401	1547 ± 1046	660 ± 186
@2Sn - Co	760 ± 1538	-1384 ± 2190	42 ± 898	-282 ± 195

@ Paired t-test --- 1. All N.S.
2. 1.25mg $p < 0.05$

All values represent mean ± standard deviation.

Number of animals in control group (n) = 4
Each ATP group (n) = 5

Flow is measured in ml/min/g.

Vascular resistance is measured in mmHg/min/g (using B1 for calculation of the cobalt value and B3 for calculation of tin value).

Table 4.8. Mean and standard deviation of blood pressure measured at the four reference points in ATP animals

			ATP µg/animal		
		Control	0.25	0.75	1.25
Blood pressure					
Cobalt	B1	149 ± 57	123 ± 24	144 ± 29	143 ± 15
Tin	B2	155 ± 57	117 ± 18	137 ± 28	144 ± 9
	B3	155 ± 57	107 ± 14	117 ± 22	100 ± 8
	B4	155 ± 57	77 ± 24	96 ± 9	85 ± 11

Number of animals in control group (n) = 4.
Each ATP group (n) = 5.

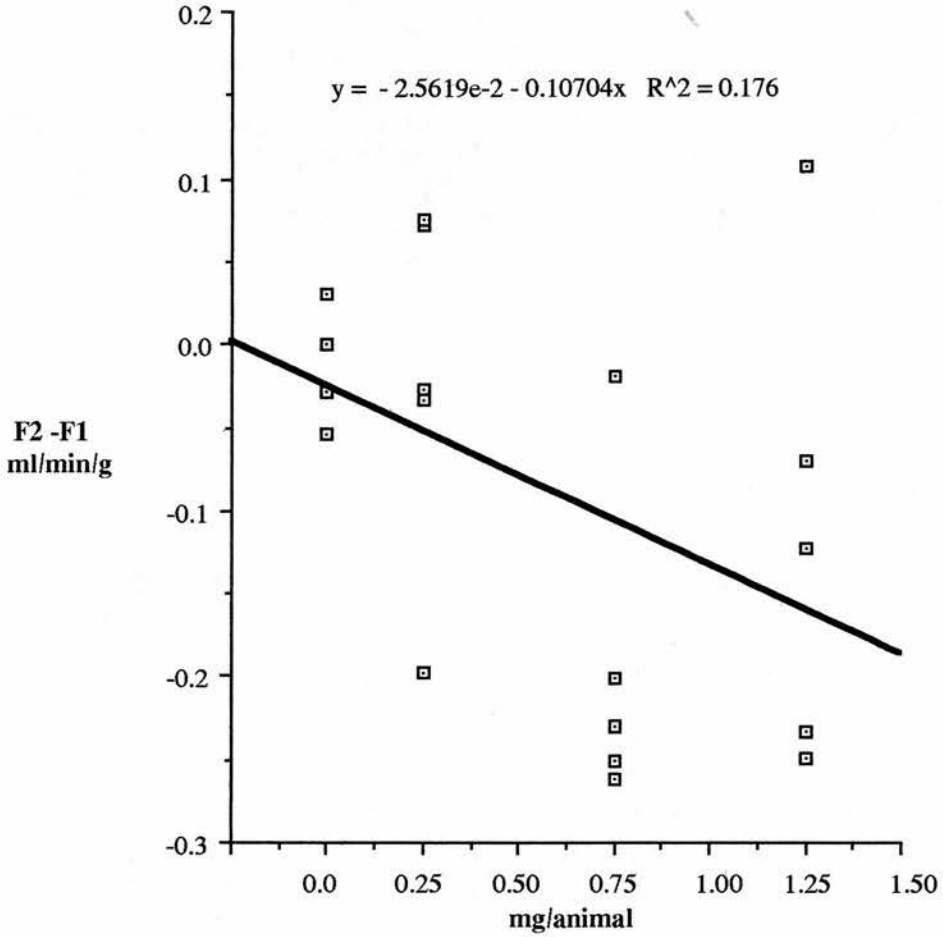
Blood pressure is measured in mmHg.

B1 and B3 Blood pressure at point of microsphere injection used for calculation of vascular resistance.

B2 Blood pressure at the begining of the experimental run, used for the calculation of blood pressure change (B4 - B2).

B4 Blood pressure at the point of maximum drug effect.

Figure 4.10. Change in Bone Blood Flow with ATP Treatment

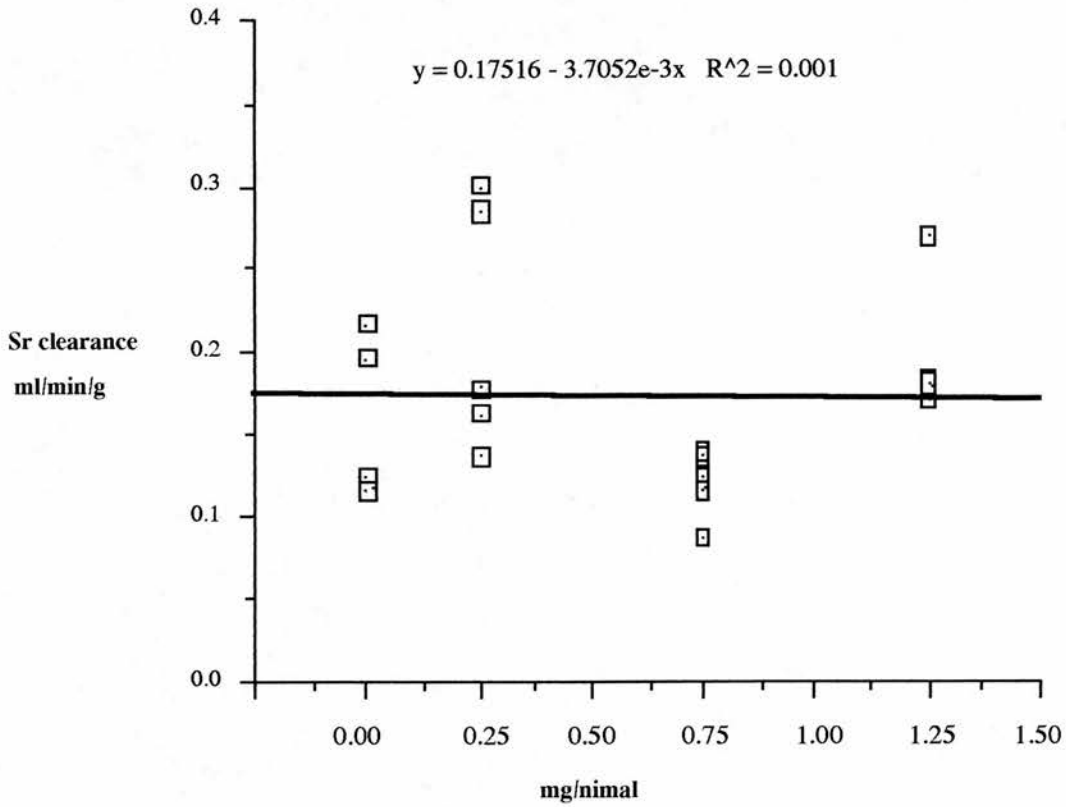


F1 = Control Blood Flow -- using Co mocrospheres.
F2 = Experimental Blood Flow -- using Tin microspheres.

Significance of the Regression, $p \leq 0.09$

Correlation coefficient (r) = - 0.41

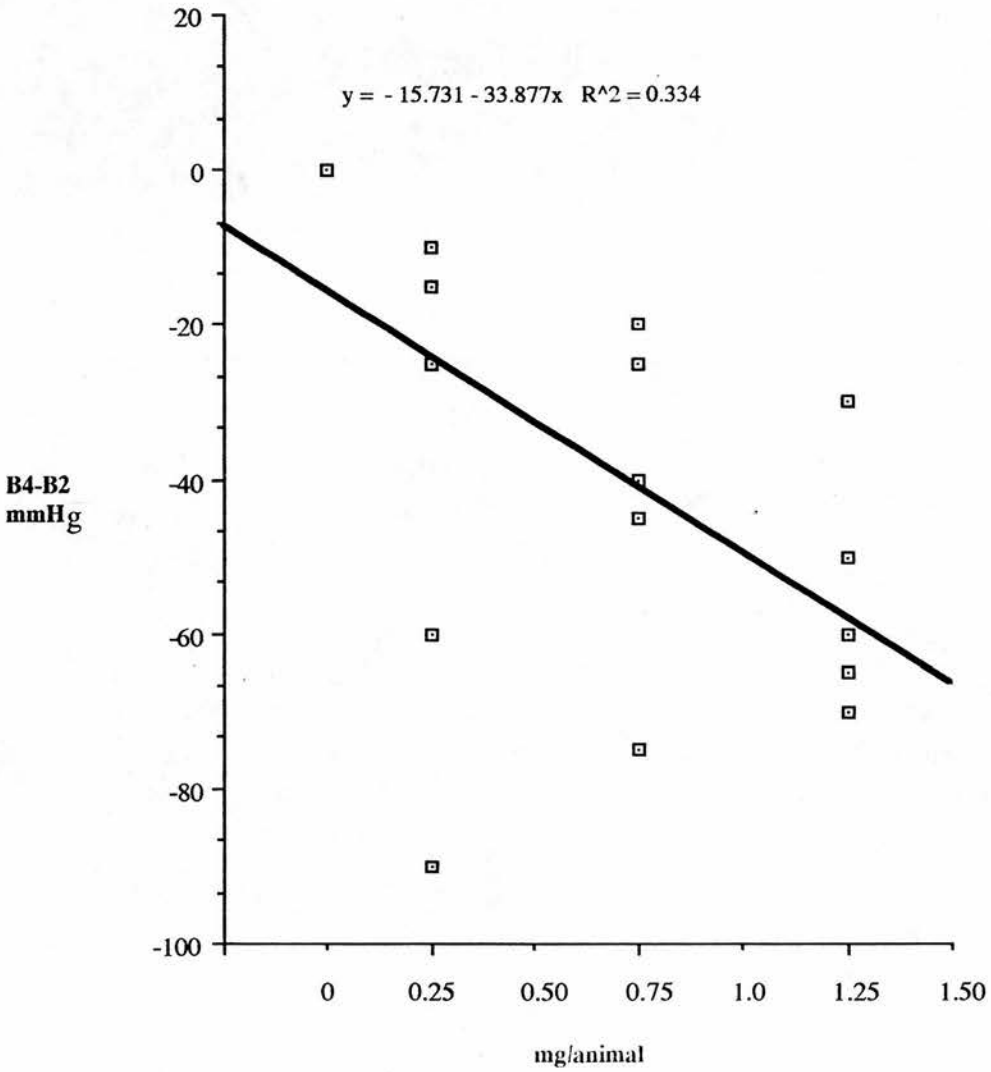
Figure 4.11. Effect of ATP on Strontium Clearance.



Significance of the Regression, $p \leq 0.9$

Correlation coefficient (r) = - 0.03

Figure 4.12. Change in Blood Pressure in response to ATP



■ Four points

B2 = Arterial Pressure measured at begining of experimental point.
B4 = Arterial Pressure measured at point of maximum drug effect.

Significance of the Regression, $p \leq 0.01$

Correlation coefficient (r) = - 0.58

Figure 4.13. An example of the arterial pressure trace from an animal given 1.5mg ATP

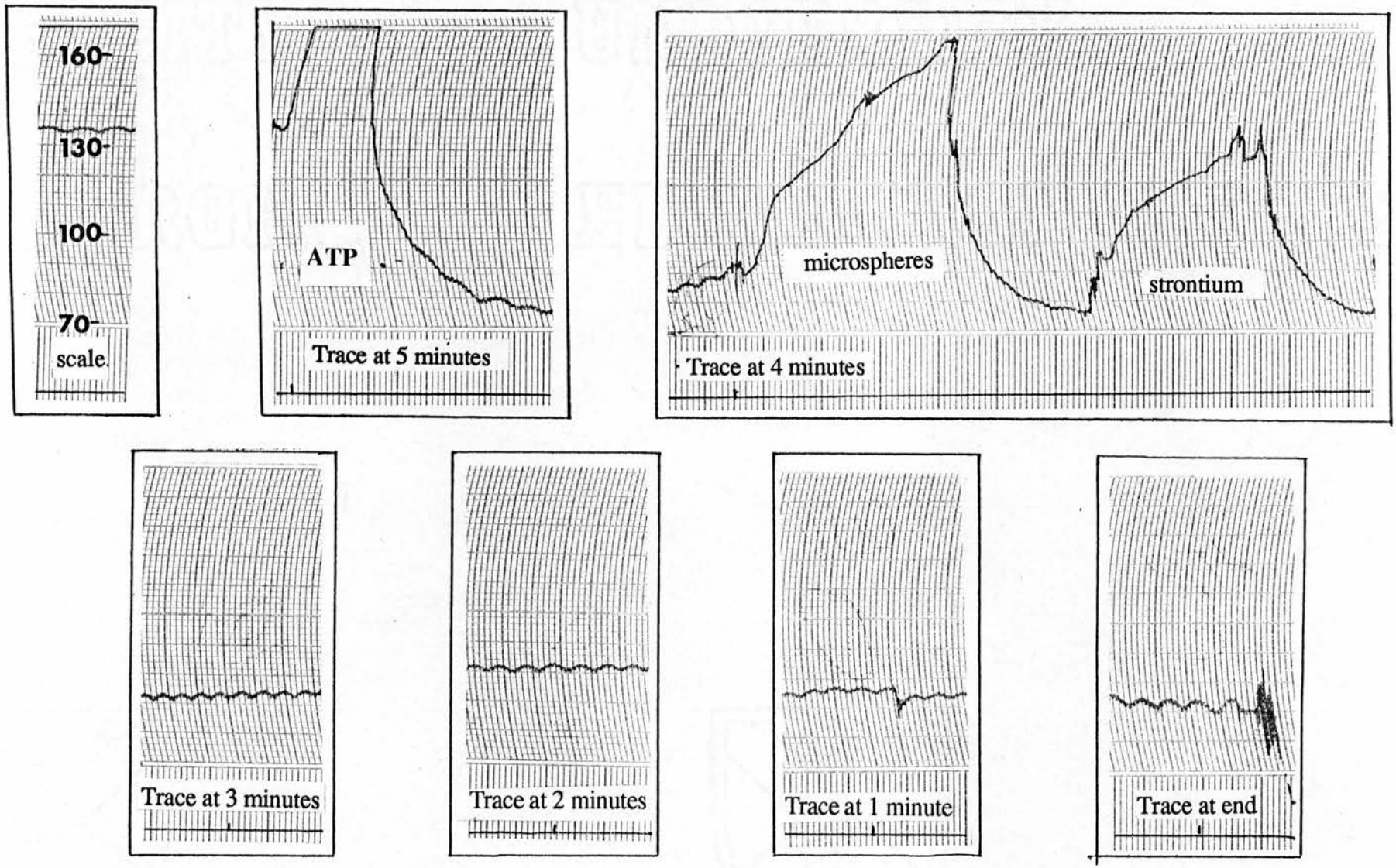
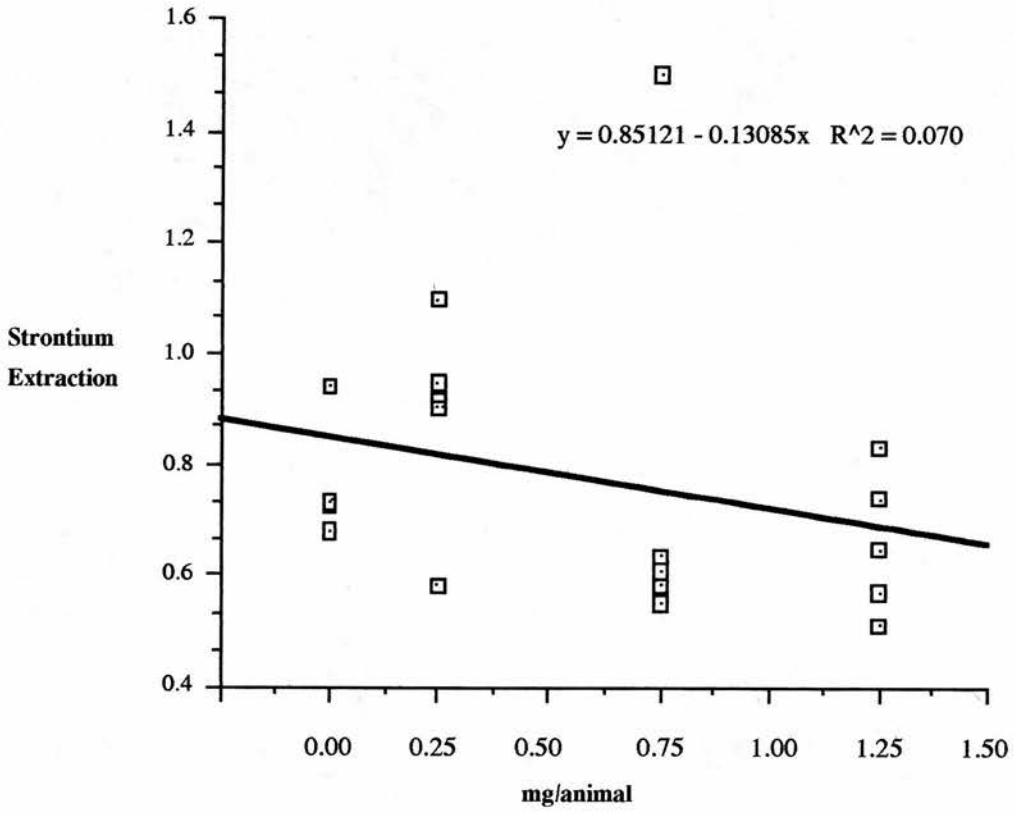


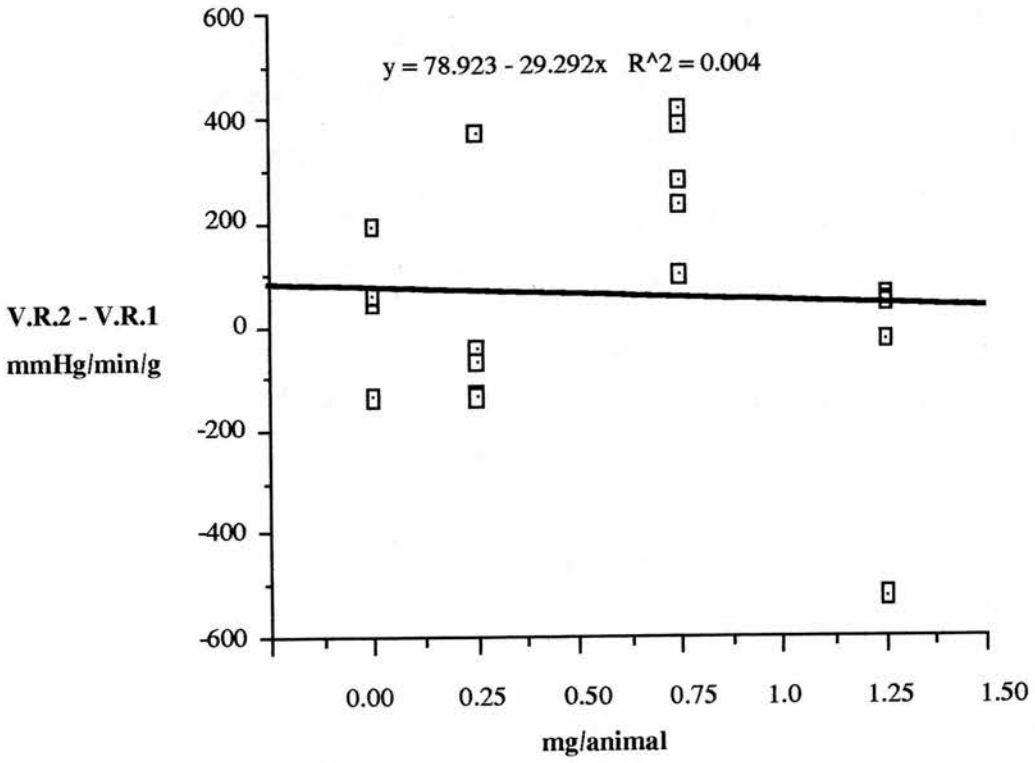
Figure 4.14. Effect of ATP on Strontium Extraction.



Significance of the regression, $p \leq 0.3$

Correlation coefficient (r) = - 0.265

Figure 4.15. Change in Bone Vascular Resistance in Response to ATP

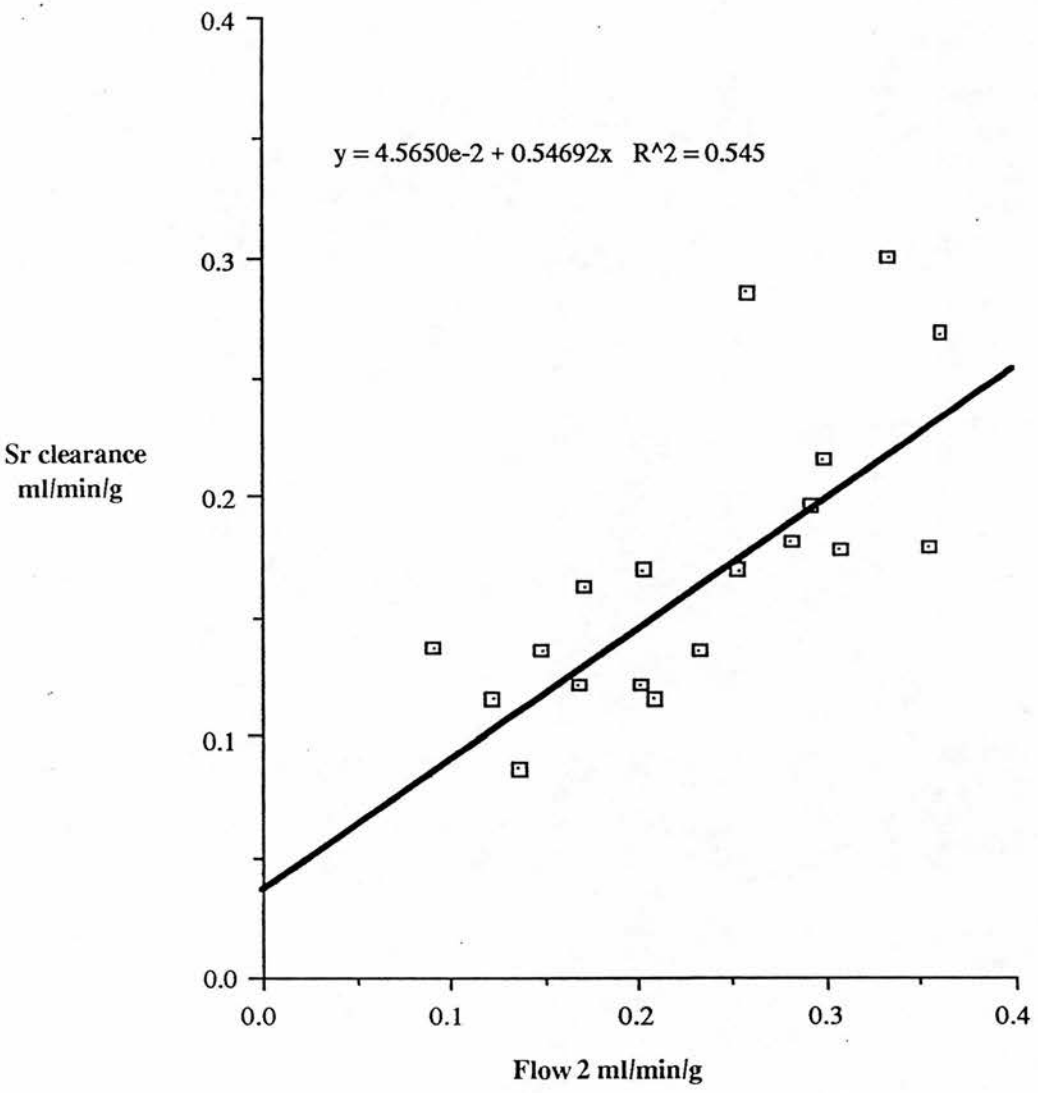


V.R.1. = Vascular Resistance as estimated from cobalt flow and blood pressure (B1).
V.R.2. = Vascular Resistance as estimated from tin flow and blood pressure (B3)

Significance of the Regression, $p \leq 0.8$

Correlation coefficient (r) = - 0.063

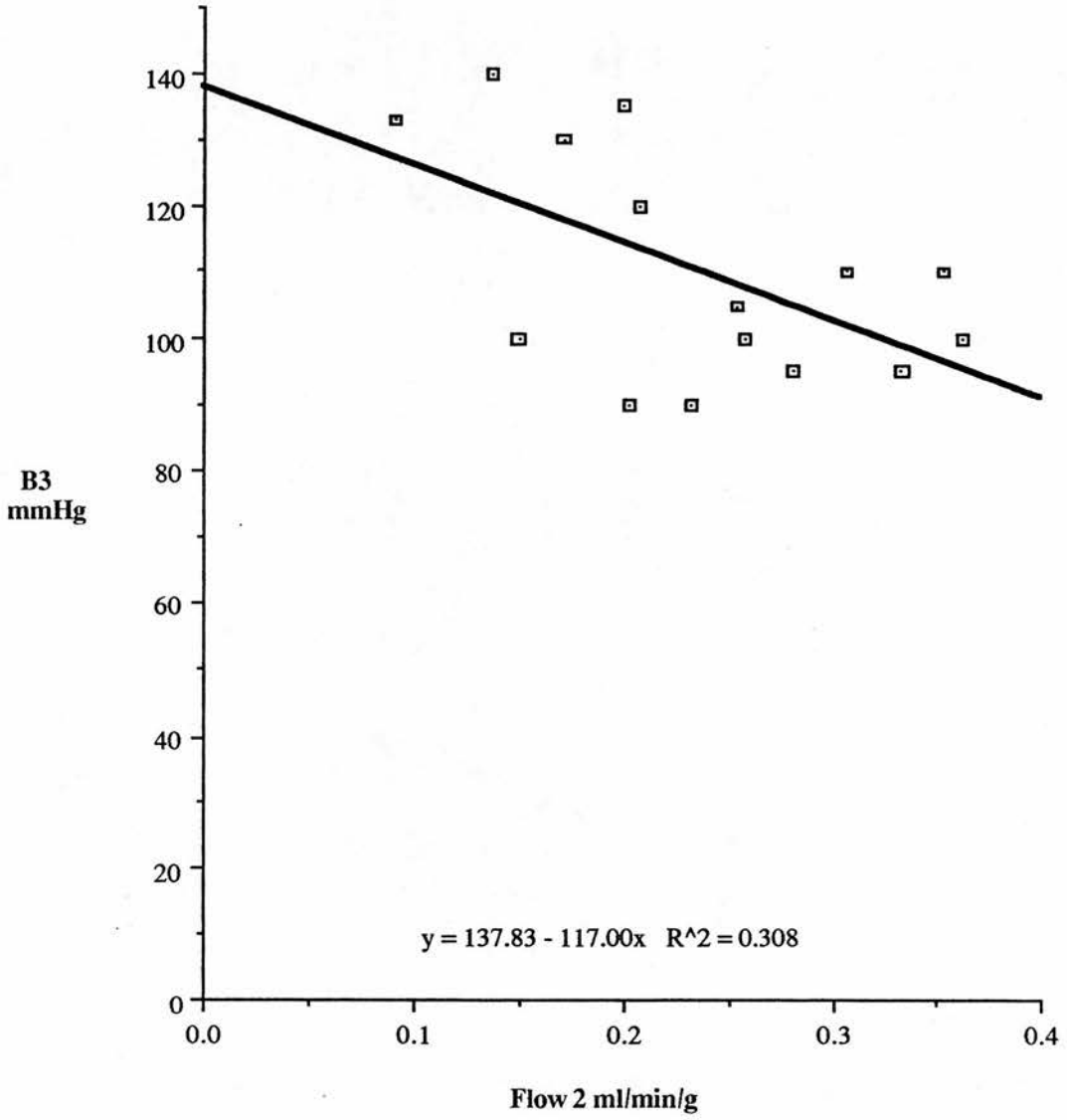
Figure 4.16. Flow Versus Strontium Clearance in ATP Treated Animals



Significance of the regression, $p \leq 0.001$

Correlation coefficient (r) = 0.69

Figure 4.17. Flow 2 Versus Blood Pressure in ATP Animals



Significance of the regression, $p \leq 0.03$

Correlation coefficient (r) = - 0.55

4.6. Discussion

4.6.1. Effect of vasoactive agents

Noradrenaline

In summary administration of noradrenaline resulted in four significant trends.

1. A decrease in bone blood flow with increasing concentration. At the maximum dose this flow is reduced by 48%.
2. An increase in arterial pressure with increasing noradrenaline concentration.
3. A similar increasing dose response in bone vascular resistance.
4. An increase in strontium extraction with increased dose of noradrenaline.

Noradrenaline appears to have no effect on the clearance of strontium.

Hypertensive action of noradrenaline

These findings are similar to those of Gross et al (1979) who noted that intravenous infusion of noradrenaline increased arterial pressure from 111 ± 4 to 136 ± 5mmHg in the dog. This change in arterial pressure represented a 25% increase and is similar to the mean percentage increase shown at the maximum dose of noradrenaline used in this study. Gross found that blood flow to bone and marrow was decreased and vascular resistance significantly affected at the same time as this pressure effect. Vascular resistance in the diaphysis of the femur increased to 237% when a 25µg/min dose was used and this is slightly higher than the increase when the 2.0µg dose was used in the rat (220%). These authors suggest that the constrictor response was a direct effect of the agent on vascular smooth muscle.

The results of this study are also similar to earlier work by McCarthy et al (1985). Noradrenaline infusion increased bone vascular resistance in a dose dependent fashion while significantly decreasing blood flow and, despite these changes there was no significant change in strontium clearance.

In an earlier paper Michelsen (1968) found flow and intramedullary venous pressure to decrease markedly in response to noradrenaline (1.6 to 4.8µg/10 sec), this resulted in an increase in the arterial resistance within the bone marrow. This author also noted that the duration and magnitude of these responses increased with infusion dose, similar to the findings of this study.

Driessens and Vanhoutte (1981) have also demonstrated that noradrenaline (2µg/ml whole bone) affects bone vasculature, the administration of the endogenous solution giving rise to constriction of the blood vessels within the perfused tibiae. A previous study by these authors (1979) examined the flow pressure relationship in an isolated dog tibiae. Injections of 0.5µg boluses of noradrenaline caused a dose dependent increase in perfusion pressure. This response was abolished by phenatolamine an alpha-adrenolytic drug, suggesting that this constriction was a result of exposure to alpha-adrenergic agonists.

Therefore the results of this work, ie. the increase in bone vascular resistance could be expected from the findings of these studies. An increase in vascular resistance would reduce blood flow and this is seen in the measured bone blood flow of the animals used here.

Effect of noradrenaline on mineral exchange

Noradrenaline does not however significantly affect strontium clearance. Clearance is dependent upon the surface area available for exchange thus the lack of effect implies that the capillary surface area remains constant. That is noradrenaline does not increase the number of patent capillaries. The extraction of strontium is however affected by noradrenaline, and is significantly increased. This variable is dependent on flow and clearance and although clearance remains relatively constant, bone blood flow is significantly decreased with increasing doses. Thus the rate of blood flowing through the patent capillaries is reduced increasing the time taken for minerals to diffuse across the walls. Therefore more strontium is extracted from the blood, although the rate of this clearance remains constant.

In summary, noradrenaline acts by increasing peripheral vascular resistance and reducing blood flow without producing changes in the functional properties of the microcirculation in bone. Its effect on strontium extraction is dependent on the change in bone blood flow and does not represent a direct action on the exchange process.

ATP

The administration of ATP resulted in one significant trend, arterial blood pressure was decreased in a dose dependent manner. The other variables, such as flow, did display dose dependent trends although none of these were statistically significant from zero.

Hypotensive action of Adenosine and ATP

It is accepted that adenosine is vasodilatator but there are few references that investigate this property in bone. Gross et al (1979) concluded, from their study of neurohumoral regulation of bone blood flow, that adenosine dilates bone and marrow vessels, thus indicating a dilatory capacity in bone and marrow smooth muscle. These authors found that in conscious and anaesthetised dogs adenosine reduced arterial pressure and significantly lowered vascular resistance while blood flow to muscle was significantly increased. In the anaesthetised animal the decrease in arterial pressure was approximately 55%, which is similar to the percentage reduction shown at the 1.25mg dose of ATP used in this study. The effect on vascular resistance demonstrated by these authors in response to adenosine was not found following ATP treatment in the rat, in fact in this study bone vascular resistance varies to a large degree with large standard deviations for the means.

McCarthy et al 1985 found a similar significant decrease in bone vascular resistance with an accompanied increase in flow in response to ATP. Strontium clearance was also included in this study and was found to remain constant during the infusion of ATP. The present results contradict to some extent the findings of other authors, a decrease is found in arterial blood pressure but no significant trend is shown in vascular resistance or bone blood flow. Thus in the rat ATP causes vasodilatation of blood vessels thereby reducing peripheral blood pressure but this is not of a sufficent magnitude to affect vascular resistance or flow. Infact in bone vascular resistance and flow remain unchanged. Analysis of flow and blood pressure shows that these variables are not correlated to any degree (r=0.55 Figure 4.17.), as was found in the animals administered

noradrenaline (r=0.3 Figure 4.9.). Vascular resistance in bone is calculated from both bone blood flow and arterial blood pressure. Here flow does decrease to some extent in response to ATP, although not significantly and this reduces the change in vascular resistance that would arise if pressure dropped and flow remained constant. The lack of significant effects on bone vascular resistance and on bone blood flow suggests that the dilatory effect of ATP is mainly on the peripheral circulation with little dilatation occurring in bone vessels. Thus the systemic vascular resistance appears to be affected by ATP but the values for muscle vascular resistance are too variable to allow any real conclusions to be drawn about its systemic effects.

Effect of ATP on mineral exchange

Like noradrenaline ATP has no significant effect on strontium clearance. This is in agreement with the work of McCarthy et al (1985). Unlike noradrenaline the administration of ATP does not however affect strontium extraction, bone blood flow remains relatively constant and therefore extraction which is calculated from both flow and clearance remains constant as well.

In summary, ATP acts on the systemic circulation reducing arterial blood pressure. It has no effect on bone blood flow or strontium clearance. Therefore in a similar way to noradrenaline, ATP does not appear to change the functional properties of the bone vascular bed.

4.6.2. Mechanism of action

Noradrenaline

The nature of the blood vessels in bone obviously plays a vital part in the response to noradrenaline. The anatomy of the blood vessels supplying the skeleton is well documented (Brookes 1971, Rhinelander 1972). The blood vessels supplying the marrow, the intramedullary arteries, have an endothelial, a muscular and an adventitial layer. After further branching within the marrow vascular bed the arteries lose their muscular component and the walls consist of only an endothelial and adventitial layer branching off into sinusoids (Michelson 1968). The active constrictor effect of noradrenaline appears to be through action on this smooth muscle component as the response of bone vessels is of similar magnitude to that of skeletal muscle (Gross et al 1979). The presence of these smooth muscle cells has been confirmed by Driessens and Vanhoutte (1979).

This study shows that bone vasculature is responsive to noradrenaline over a range of concentrations and that this is in a dose dependent manner. Several workers suggest the presence of intraosseous vascular alpha receptors (Yu et al 1972) and the study of Tran et al (1978) agrees with these findings highlighting the presence of alpha-adrenoreceptors. Here noradrenaline reduced blood flow and simultaneously increased arterial blood pressure and bone vascular resistance, the most obvious conclusion is that it acts directly on the bone vasculature, possibly at local receptor sites, to reduce bone blood flow through an increase in resistance. It appears that the constrictor effect of noradrenaline opposes or masks any increase in flow that would normally arise through an increase in arterial perfusion stimulating the arterial baroreceptors. This further supports the theory of local receptor sites for noradrenaline which controls its action in bone. Analysis of flow and blood pressure shows that the two variables are to a limited extent correlated (Figure 4.9.), though the actual values are scattered. This suggests that

a change in flow does cause a change in arterial blood pressure and the drug appears to be responsible for both actions. The use of alpha-adrenolytic drugs confirms that the action of noradrenaline is through adrenergic receptors present locally in the smooth muscle cells of the arteries.

ATP

The vasodilatory action of ATP is probably through its action on the smooth muscle cells of blood vessels, like noradrenaline the magnitude of the response of bone vessels is similar to that of smooth muscle Gross et al(1979). McCarthy et al(1985) found that bone did respond to ATP, vasodilatation reducing bone vascular resistance. This however was not seen in the presented results. It has however been shown that alkaline phosphatase is present in the bone osteoblasts. This enzyme may have been hydrolyzing ATP before the bone vascular bed could respond, thus no dilatatory response was seen. Malik (1987) advanced the theory that stimulation of the sympathetic nerves can result in a the release of adenine nuclides, these in turn enhance the synthesis and release of prostaglandins from several tissues. Thus the action of ATP on vascular smooth muscle may be influenced by these prostaglandins.

4.6.3. The relationship between flow and clearance

The plot and analysis of flow versus strontium clearance in the drug treated animals compared with the control group shows that neither noradrenaline nor ATP are statistically different from the control or from one another. Since clearance appears to be related to flow this suggests that these act through a vascular mechanism either directly or indirectly, any change in flow reflected by a change in clearance. The presence of intraosseous alpha-adrenoreceptors and the inhibition of noradrenaline action by an alpha-adrenolytic drug suggests that a direct vascular mechanism is involved in the constrictor response. The action of ATP appears to be on the general

circulation, no effect is shown on bone blood flow or bone vascular resistance even though arterial blood pressure is reduced. This effect may be influenced by the release of prostaglandins that occurs after stimulation of the sympathetic nerves.
The lack of a change in strontium clearance indicates that this constrictor/dilator action does not involve increasing the number of patent capillaries.

4.7. Conclusion

Noradrenaline reduces blood flow by increasing the peripheral vascular resistance, vascular receptors present in blood vessels respond to the drug resulting in vasoconstriction. Although bone vessels appear to respond in a similar manner no significant effect is shown on strontium clearance suggesting that the number of patent capillaries remains the same i.e. the surface area for diffusion is constant. The small changes in clearance reflect the relationship between flow and mineral transport rather than a direct action of drug on exchange. On the other hand ATP reduces arterial pressure through vasodilatation of the general circulation but has no effect on blood flow or vascular resistance in bone. Like noradrenaline it has no effect on strontium clearance and similarly ATP does not change the functional properties of the vascular bed.

CALCIUM REGULATING HORMONES

5.1. Introduction

The calcium regulating hormones have been shown to control calcium and other ion levels in the plasma through their action on bone. The movement of these ions i.e. calcium, phosphorus and magnesium in and out of the skeleton has been extensively studied. The use of isotopic tracers such as 47-Ca allows attempts to be made at quantifying intestinal absorption, skeletal accretion and resorption rates. However little work has been attempted to identify how agents affecting mineral levels in the blood act on the vascular bed of bone, even though studies of circulation in other beds suggest that bone circulation is in some way influenced.

Both PTH and PGE2 have been demonstrated to have hypotensive action in vascular beds,vasodilatation of vessels reducing blood pressure (Charbon 1968, Messina et al 1976). Evidence is also accumulating that PTH influences bone vascular resistance, Pang et al (1980a) found that bPTH had a hypotensive effect in the rat. Additionally these agents have been shown to have rapid inhibitor effects on calcium uptake (Dacke and Shaw 1987), the authors suggesting that a vascular response to PTH and PGE2 may be involved in this inhibition mechanism. Elevation of plasma calcium levels above the normal range is referred to as hypercalcemia. Several authors have investigated this action with respect to PTH and PGE2, in particular examining the timing of the response (Boelkins et al 1976, Beliel et al 1973). Both the hypercalcemic and the histological effect of PTH and PGE2 have been more extensively studied than the vascular action. Thus there are few studies which detail the vascular effect of these agent with respect to mineral movement in bone.

Calcitonin has been shown to have opposite action to that of PTH and PGE2 i.e. causing hypocalcemia (a reduction in plasma calcium levels below normal). Johnston and Deiss (1966) established that this agent has inhibitory effects on elevated plasma calcium levels. This is through complex interactions between the agents rather than inhibition

of the PTH or PGE2 function. There is ample evidence that calcitonin inhibits bone resorption (Foster et al 1966) however like PTH and PGE2 its effect on blood flow is not so well documented.

5.2. Choice of drug and dosage.

The general action of PTH, PGE2 and calcitonin has been well documented however the papers are often contradictory, especially in the case of PGE2, and few have included a study of their effects on blood flow and mineral clearance in bone. These papers provide details of effective doses that are suitable for use in the rat thus allowing selection of doses which should have some effect either on the arterial pressure or strontium clearance.

It has been established that PTH induces hypercalcemia and it seemed appropriate to investigate the action of this on bone blood flow as well as calcitonin, whose effects oppose PTH's action. PGE2 has also been included because it has been shown to have a rapid inhibitory effect on calcium uptake in bone. The doses of PTH and PGE2 were selected from work by Dacke and Shaw (1987). The doses of PGE2 used by these authors are comparable with the lowest doses of native PGE2 found to produce osteolytic responses in vitro (Raisz and Martin, 1984 -- 1-10nmol/l). The administration of approximately 2,10 and 20µg/100g body weight of rat decreased net uptake of 45-Ca in the femur in a dose dependent manner. Only at the highest dose was a significant difference found from the control values. In a 350g animal these doses are equivalent to 7, 35 and 70µg respectively. For ease in preparation and in the creation of a dose curve the lowest selected dose in this study was chosen as 20µg increasing to 80µg in increments of 20. Thus doses similar to these authors were used but the highest dose increased by 10µg.

In investigating the effects of bPTH(1-34) these authors used approximately 0.3, 1.5 and 3.3µg/100g (Dacke and Shaw 1987 Figure 1.). These caused a decrease in calcium-45

uptake which was significantly different at all doses. The doses are equivalent to 1, 5.25 and 12µg in a 350g rat. As all the doses were statistically significant it was decided to select doses which fell within this range i.e. 1-12µg and create a dose response curve from these. Once again for ease in preparation whole microgrammme quantities were selected thus the doses chosen were 4, 8, and 12µg per animal.

The doses of calcitonin were selected from a study conducted by Driessens and Vanhoutte (1979). These authors used doses ranging from 0.001 to 1mU. of salmon calcitonin perfused over 15 minutes into a dog tibia . They found at all doses the results were statistically different from the control results. The two highest doses were equivalent to 10U. and 100U. and it was decided to select 10U. as the starting dose for infusion into the rat (This was the same as the selected dose of Porter et al , per gramme of bone). The next dose of 50U. was then chosen. Then a 25U. dose was selected as a intermediate point to give a dose response pattern. Thus the three selected doses would hopefully cause some effect on vascular perfusion pressure which Driessens et al found to be affected in the isolated bone.

Circulating levels of the hormones

Little is known about the basal secretion of the endocrine glands in situ since the procedures tend to involve tropic stimulation to some extent thus upsetting the secretion rates. The measurement of circulating basal levels of these hormones is also hard to establish but has been attempted through histochemical bisoassays and radioimmunoassays. However PGE2 is secreted locally, the level changing to suit the required needs. Using histochemical bioassays the circulating concentration of PTH in the human was found to be approximately 5 - 15pg/ml of plasma (Williams 1981a). Another method (cytochemical bioassay) found that the mean value for a normal subject was 16.8 ± 3.6pg/ml of plasma. The lower values of this study would therefore fall within the range of the histochemical assay. However this method (cytochemical) measures all the immunoreactive PTH in the peripheral plasma i.e. the inert metabolites as well as the

active portion. The histochemical assay is more representative of the circulating level of the active form of PTH and since bPTH (1-34,) the active portion is used in this study this quantity would reflect the dose better. Thus assuming a 30ml plasma volume for the rat the doses used in this study range from 0.13µg to 0.4µg and represent pharmacological levels.

Calcitonin levels have been measured successfully with numerous techniques but the radioimmunoassay method is the most accurate and sensitive reflecting the specificity of the hormone. Parthemore et al (1978) have found that in a normal male the mean value for plasma calcitonin is 24pg/ml ± 18pg with a range of <10 - 75 pg/ml for 55 males. These findings agree with those of other laboratories, thus the basal circulating levels of calcitonin in normal adults are low. Although other authors have found higher levels (Heynen et al 1976) this could arise because of the immunochemical heterogeneity of calcitonin or the method of measurement (i.e. histochemical assays). Therefore in the normal rat (30ml plasma) the doses administered represent pharmacological levels i.e. 0.08µg - 4.2µg

5.3. Preparation of Drug

Before the experimental procedure the various drugs used were prepared to ensure activity and 'freshness'. In each drug group the dose used was randomly chosen to try to reduce variation due to different sibling groups. In all cases a stock solution of the drug was either bought or made up and then stored at the appropriate temperature. An appropriate volume was then removed when required and diluted with Krebs Ringer buffer to give the necessary concentration.

a) Parathyroid Hormone (Sigma bPTH(1-34) 0.1mg.)-- 4,8 and 12ug per rat 2ml bolus. Since the structural requirements for most of the biological activities of parathyroid hormone (PTH(1-84)) are virtually satisfied by the amino-terminal 34 amino acid fragment, PTH(1-34) this study use bPTH(1-34) to investigate the action on bone (Tregear et al 1973).

The stock solution was made up by dissolving 0.1mg bPTH 1-34 in 2ml saline which was then stored at <0°C . For each animal the test solution was made up when required. Thus for a dose of 4µg, 0.08ml of the stock solution was dispensed and this was made up to a volume of 2ml using buffer. (8µg -- 0.16ml, 12µg --0.24ml).

b) Prostaglandin E2 (Sigma) -- 20,40,60 and 80ug per rat 2ml bolus.

Using 1000µg PGE2 and 0.5ml ethanol a stock solution was made. This was stored at less than 0°C and the appropriate volume dispensed when required. Thus for the 20µg dose 0.02ml of the stock solution was required with 0.04, 0.06 and 0.08ml required for the other doses. These quantities were then made up to 2ml with buffer containing 0.1% albumin.

Since more than one animal was experimented on in any one day larger volumes than these were actually dispensed i.e. for required doses of 20,40 and 60µg 0.12ml were measured. These were then made in to the 60µg concentration using buffer i.e. made up to 4ml volume. Two ml of this solution was then dispensed for the 60µg dose, 1.33ml for the 40µg dose and 0.66ml for the 20µg dose both of these then made up to 2ml using buffer.

The quantity of ethanol therefore varied depending on dose but this addition was essential, as the ethanol acted as a 'carrier' for PGE2. To ensure that it was not the ethanol that caused any effect the control animals also received ethanol in the injected buffer at the same concentration as that of the 80µg animals i.e. 0.04ml/ml of buffer.

c) Calcitonin (Rorer; Calysnar 400U. of Salmon Calcitonin in 2ml of saline actate diluent) -- 10,25 and 50U. per rat infusion, stored between 2°C and 8°C.

After a test injection of Calysnar 10U. which displayed no effect on flow or arterial pressure the protocol was changed to incorporate infusion of the drug. This was therefore similar to the method used in the perfusion of a dog tibiae (Driessens et al 1979, Porter et al 1986)

To ensure that the animal received the same quantity of solution as for PTH and PGE2 the pump rate was increased to 15ml/hour and the period extended to eight minutes. Test

infusions of buffer over this period and at this rate showed no difference when compared to the other control animals.

The Calysnar solution was 200IU.per ml. Thus to obtain the correct concentration for the test animal quantities of this was dispensed and diluted with buffer. To maintain efficient infusion rates more than 2ml of the solution was made up i.e. 6ml quantities were for two rats. Thus for the 10U. dose 0.15ml was dispensed and made to six ml. (0.375 in 6ml for two doses of 25U. and 0.75ml in 6ml for two doses at 50U)

5.4. Experimental procedure (Figure 5.1.)

The animal was prepared for the experiment as detailed in section 4.4.

a) PTH and PGE2

1. Control blood flow.

Buffer was infused at 6ml/hour over the control flow period, this ensured that the carotid cannula remained patent over the experimental period. Withdrawal of blood commenced (caudal cannula 0.197m/min) and thirty seconds later Co-57 microspheres were injected into the heart via the first tap. Blood was withdrawn for a further ninety seconds.

2. Test blood flow

After ensuring that arterial blood pressure was stable the test run was begun. The test solution was injected via the carotid cannula and thirty seconds after this was begun blood collection was commenced. After a further thirty seconds tin-113 microspheres and strontium-85 were injected. Blood withdrawal ceased after a total of 4.5 minutes.

b) Calcitonin

1. Control blood flow

The same procedure was followed however buffer was infused at 15ml/hour.

2. Test blood flow

The buffer was replaced with the test solution and infusion begun (carotid cannula 15ml/hour). After three and a half minutes the pump for withdrawing blood was started (caudal cannula 0.197ml/min). Thirty seconds later the microspheres and strontium were injected. Blood withdrawal ceased four minutes later.

c) Control

1) PTH and PGE2

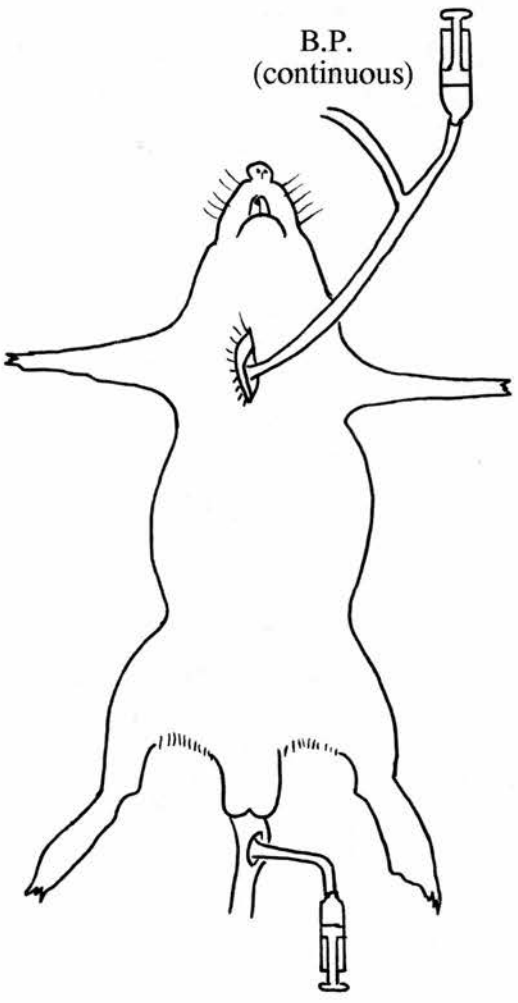
The same procedure was followed except that a bolus injection of buffer,via the carotid cannula, was used instead of the test solution . Ethanol and albumin was included in the buffer for the PGE2 animals.

2) Calcitonin

The same procedure was followed as for the calcitonin treated animals i.e. infusion of buffer over eight minutes at 15ml/minute.

At the end of the experiments the animals were sacrificed using KCl injected via the tail cannula. Both tibiae and femora were removed quickly to minimise post-mortem migration of ions to the bone. A muscle sample from the upper quadriceps was also taken. The bone, muscle and blood samples were then weighed before counting.

Figure 5 .1. Schematic Presentation of Procedure



Infusion pump

To carotid artery:

1. Co-57 μspheres.
2. Drug (per animal)
   a. INFUSION
      Calcitonin 0, 10, 25 and 50U.
      (0, 0.74, 1.8, and 3.6mM..)
   b. INJECTION
      PTH 0, 4, 8 or 12μg
      (0, 1, 2, and 3μM.)
      PGE2 0, 20, 40, 60 or 80μg
      (0, 0.06, 0.12, 0.18, and 0.24mM.)

Sn-113 μspheres
Sr-85.

Withdrawal pump

From caudal artery:

1. 2 minutes collection
2. 4.5 minutes collection

a) Parathyroid hormone

This group consisted of 22 animals, injected with 0, 4, 8, or 12µg parathyroid hormone. The effect of this administration on seventeen animals is detailed in tables 5.1-5.4.. Table 5.1. includes the effect of infusion on bone blood flow, strontium clearance and arterial blood pressure. While Table 5.2. gives details of strontium extraction and vascular resistance in bone. The actual measurements of arterial blood pressure are outlined in Table 5.3. Finally the action of PTH on muscle are included in Table 5.4. Of the remaining five animals one died during the experiment, in three there were insufficient microspheres collected in either of the blood samples, and in another the withdrawal pump setting was found to be too high.

The administration of parathyroid hormone causes a significant decrease in both bone blood flow and arterial blood pressure (Figures 5.2. and 5.4.) with an associated decrease in bone vascular resistance at the first two doses. The general trend is of a decrease in both flow and pressure with increasing drug concentration. In the case of flow, at none of the individual doses is there a significant change in flow when compared to the zero dose, although the line of regression for all the values is significantly decreased from zero. The mean tin flow at 4 and 8µg are actually greater than the control mean value (Table 5.1.). Thus the plot of these means would initially rise then fall, but the values are not significantly different from the control so the general trend still stands. Blood pressure changes significantly at each dose ($p<0.02$), falling to 68% at 4µg, 55% at 8µg and 52% at 12µg (Table 5.3.).Seventy five percent ($r^2=0.75$) of the variation can be accounted for by the changing dose of PTH, indicating the extent of the relationship between the two. Table 5.3 outlines the actual values for arterial pressure measured at the four points of interest. Although B1 and B2 vary to some extent this is not statistically significant at any dose. The change in arterial pressure is immediate and still present at the point of microsphere injection (Figure 5.5.). Thus the values for B3 and

B4 are the same. Apart from the degree of change, the period of recovery varies according to dose. Examination of the pressure traces for the individual animals reveals that at 4µg the trace has returned to normal by about three minutes, this is extended to four and a half minutes for the 8µg dose and in the case of 12µg the pressure does not return to normal levels before the end of the experiment. This systemic pressure decrease points towards the vasodilatatory action of the hormone, which causes a decrease in the total peripheral resistance. At 12µg the ECG trace and cardiotac, (Figure 5.6..) show changes which represent an increase in the heart rate. From this increase in heart rate and arterial pressure an increase in cardiac output can be inferred. However the radioactivity of the microspheres were not measured before injection thus cardiac output could not be estimated. This change in heart rate is in no way sufficient to compensate for the pressure drop, though there is a small increase in the pressure over the experimental period.

Bone vascular resistance shows a different pattern, the mean values suggest an initial decrease at 4 and 8µg followed by a return to approximately normal at the 12µg dose (Table 5.2.). Indeed at both 4 and 8µg there is a significant difference when analysed using a un-paired t-test. Using the line of linear regression the two variables are not correlated (r=-0.39), the injections causing only 15% of the variation in vascular resistance.

Like noradrenaline, PTH significantly reduced flow but had no significant effect on clearance. The line of regression for strontium clearance (Figure 5.3.) is not significantly different from zero and at no particular dose is any statistical difference found between the control and experimental flows (Table 5.1.). Similarly at no dose group is strontium extraction significant from the control group indicating that, although flow is decreased, this is insufficient to affect strontium extraction while strontium clearance remains relatively constant (Table 5.2.). This is, however, different to noradrenaline. Extraction is dependent on flow and clearance and a comparison of the lines of regression for flow change (Figures 5.2. and 4.2) shows that the two are significantly

different (t= 2.1 DF=29 $p<0.5$). The slope of the regression line for noradrenaline is steeper than that for PTH and since, in neither case is strontium clearance significantly affected, this would indicate that with PTH the decrease in flow is insufficient to significantly increase the quantity of strontium removed in the passage of blood through the bone capillary bed. However the greater reduction in flow following noradrenaline is sufficient to increase the extraction of strontium.

Neither muscle flow or vascular resistance are affected by parathyroid hormone. Both change in response to treatment but the standard deviations of the means are high and so no dose is significantly different statistically (Table 5.4.).

Finally, analysis of flow versus strontium clearance in the 4-12 µg animals (Figure 5.9.). There is a high correlation between the two variables, with some clumping at the lower values. Comparison of the slope with that of the control group produces a non significant t value (Figure 3.9.). Thus this relationship is only the same as that shown by normal animals and not the result of parathyroid treatment. A change in flow causes a respective change in strontium clearance, but this change in clearance need not be significant if the reduction in flow, although significant, is not too great.

Table 5.1. Effect of parathyroid hormone on blood flow, strontium clearance and blood pressure in rat bone

		PTH µg/animal		
	Control	4	8	12 #
Flow				
Cobalt	0.27 ± 0.08	0.23 ± 0.10	0.31 ± 0.07	0.33 ± 0.08
Tin	0.30 ± 0.09	0.33 ± 0.06	0.31 ± 0.015	0.17 ± 0.12
@Sn - Co	0.02 ± 0.01	0.09 ± 0.11	-0.01 ± 0.08	-0.16 ± 0.16
*** Sr clearance**				
	0.17 ± 0.02	0.19 ± 0.03	0.21 ± 0.05	0.12 ± 0.06
Blood Pressure Change				
(B4 - B2)	0	-42 ± 13	-60 ± 8	-64 ± 15

@ Paired t-test --- 0 $p \leq 0.05$

* Unpaired t-test (comparison with 0 dose) --- All N.S.

All values represent mean ± standard deviation.

Number of animals in each group (n) = 4.

\# (n) = 5.

Flow and clearance are measured in ml/min/g.

Blood pressure is measured in mmHg; at two points;

(B2) at the begining of the experimetal run,

(B4) at point of maximum effect.

Sn = Tin. Co = Cobalt.

Table 5.2. Effect of parathyroid hormone on strontium extraction and vascular resistance in rat bone

		PTH µg/animal		
	Control	4	8	12#
***Strontium Extraction**				
	0.59 ± 0.12	0.59 ± 0.05	0.69 ± 0.19	0.75 ± 0.19
Vascular Resistance				
Cobalt	521 ± 200	649 ± 277	450 ± 70	413 ± 84
Tin	415 ± 153	281 ± 79	245 ± 38	542 ± 246
@Sn - Co	-106 ± 78	-368 ± 345	-205 ± 72	129 ± 297

@ Paired t-test --- 8µg $p \leq 0.02$

* Unpaired t-test (comparison with 0 dose) --- All N.S.

All values represent mean ± standard deviation.

Number of animals in each group (n) = 4.

\# (n) = 5

Vascular resistance is measured in mmHg/min/g (using B1 for calculating the cobalt value and B3 the tin value).

Sn = Tin. Co = Cobalt.

Table 5.3. Mean and standard deviation of blood pressure measured at the four reference points in parathyroid hormone treated animals

			PTH µg/animal		
		Control	4	8	12#
Blood pressure					
Cobalt	B1	130 ± 12	132 ± 21	137 ± 10	132 ± 18
Tin	B2	119 ± 29	135 ± 18	135 ± 10	136 ± 22
*	B3}				
	}	119 ± 29	92 ± 25	75 ± 13	72 ± 10
	B4}				

Number of animals in each group (n) = 4.

(n) = 5.

Blood pressure is measured in mmHg.

B1 and B3 Blood pressure at point of microsphere injection used for calculation of vascular resistance.

B2 Blood pressure at the begining of the experimental run, used for the calculation of blood pressure change (B4 - B2).

B4 blood pressure at the point of maximum drug effect.

* Blood pressure at microsphere injection is equivalent to point of maximum drug effect.

Table 5.4. Effect of parathyroid hormone on blood flow, and vascular resistance in rat muscle

		PTH µg/animal		
	Control	4+	8+	12#
Flow				
Cobalt	0.07 ± 0.01	0.11 ± 0.05	0.11 ± 0.05	0.16 ± 0.10
Tin	0.01 ± 0.03	0.14 ± 0.04	0.35 ± 0.17	0.35 ± 0.30
@Sn - Co	0.03 ± 0.04	0.04 ± 0.08	0.23 ± 0.16	0.19 ± 0.31
Vascular resistance				
Cobalt	1821 ± 433	1354 ± 627	1445 ± 775	1095 ± 540
Tin	1135 ± 214	686 ± 316	310 ± 293	333 ± 213
@Sn - Co	-686 ± 395	-668 ± 848	-1131 ± 798	-762 ± 542

@ Paired t-test --- **Vascular resistance** 12µg $p \leq 0.05$.

All values represent mean ± standard deviation.

Number of animals in control group (n) = 3.

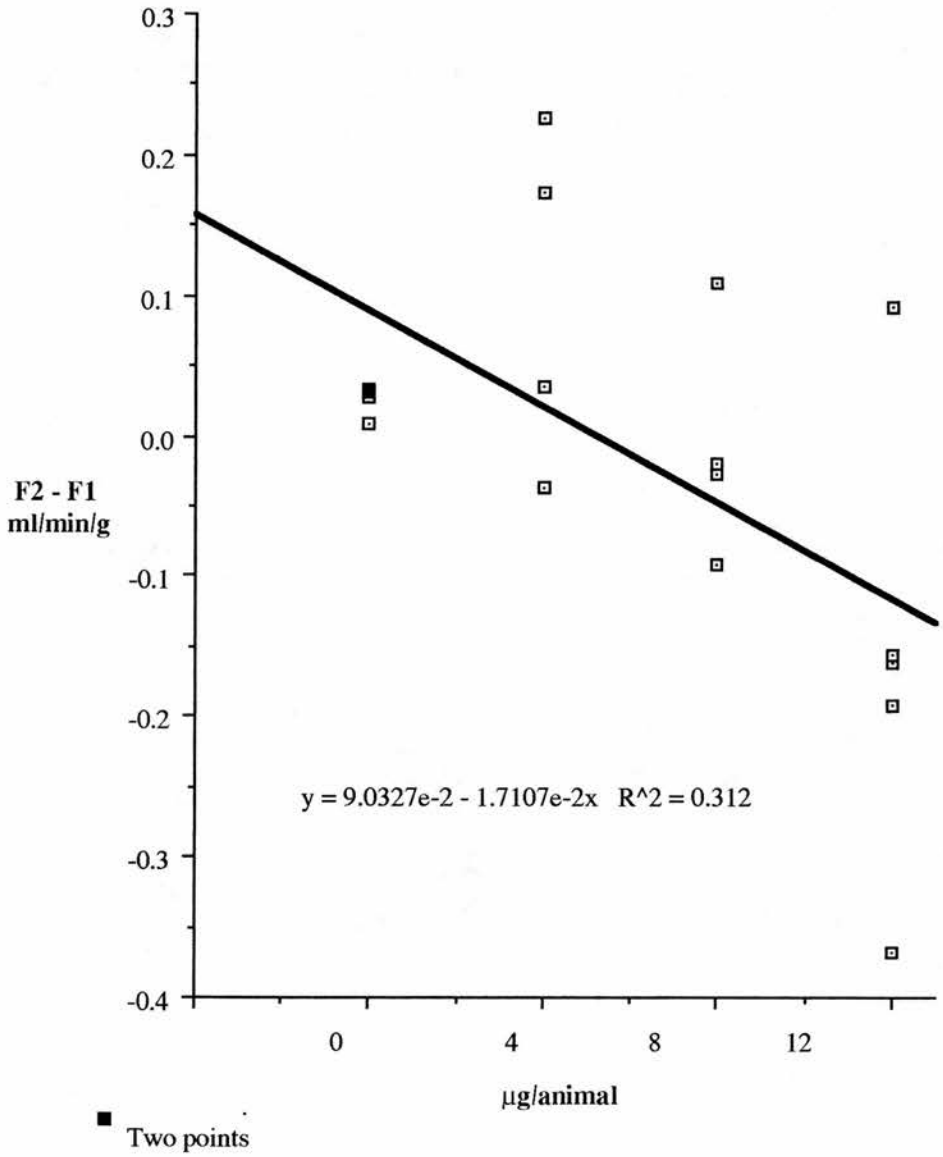
PTH treated group + (n) = 4.

\# (n) = 5.

Flow is measured in ml/min/g.

Vascular resistance is measured in mmHg/min/g (using B1 for calculation of cobalt value and B3 for calculation of tin value)

Figure 5.2 Change in bone blood flow after injection of PTH

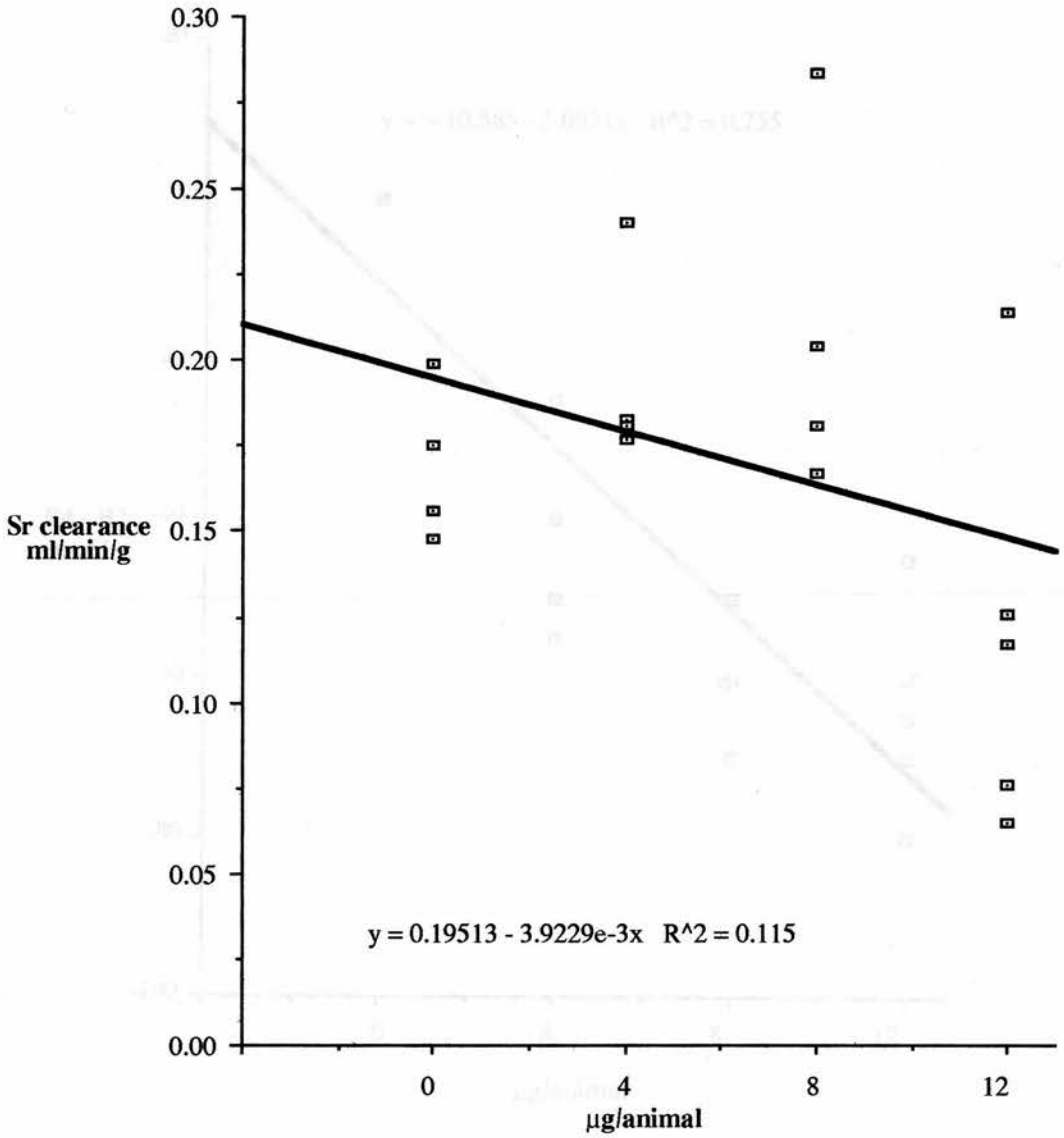


F1 = Control Blood Flow -- using Co microspheres.
F2 = Experimental Blood Flow --- using tin microspheres.

Significance of the regression , $p<0.02$

Correlation coefficient (r) = - 0.56

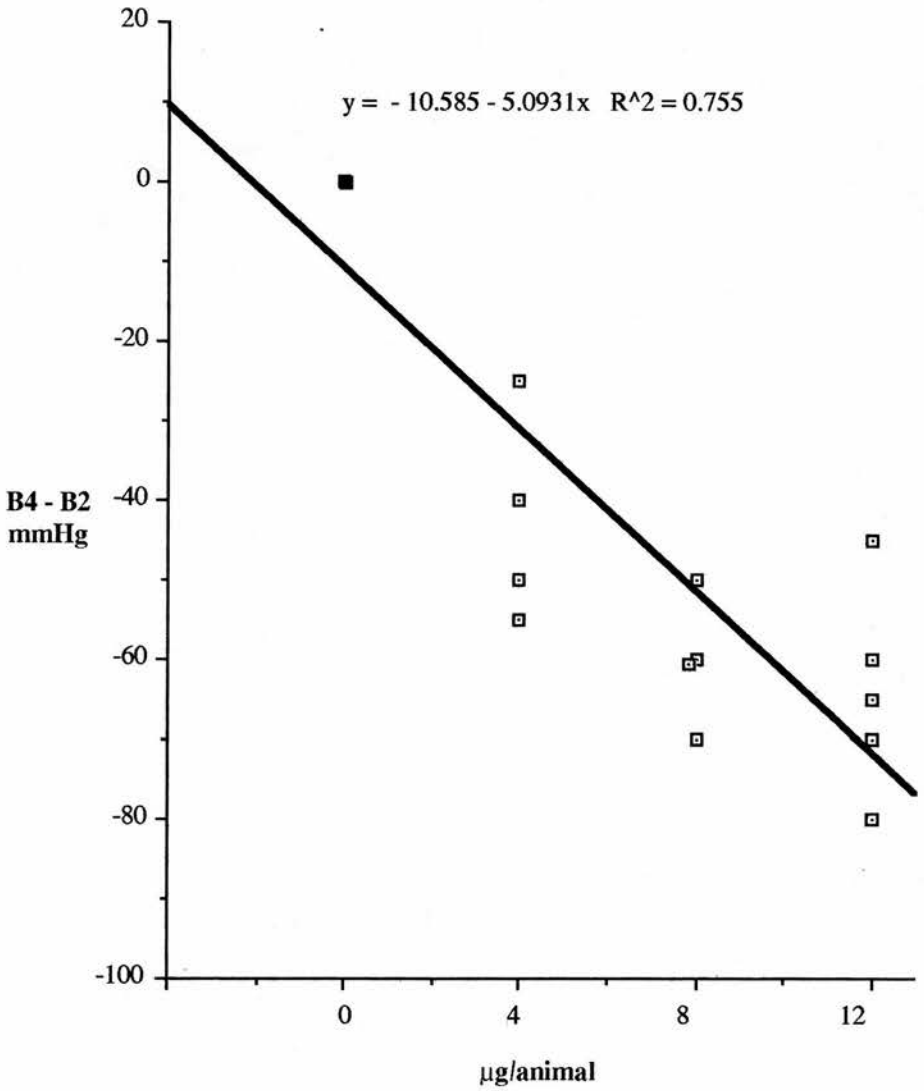
Figure 5.3 Effect of PTH on Strontium Clearance.



Significance of the regression, $p \leq 0.18$

Correlation coefficient (r) = - 0.34

Figure 5.4. Change in blood pressure in response to PTH



■ Four points

Significance of the regression , $p \leq 0.0001$

Correlation coefficient (r) = - 0.87

Figure 5.5. An example of the arterial pressure trace from an animal given 12μg PTH

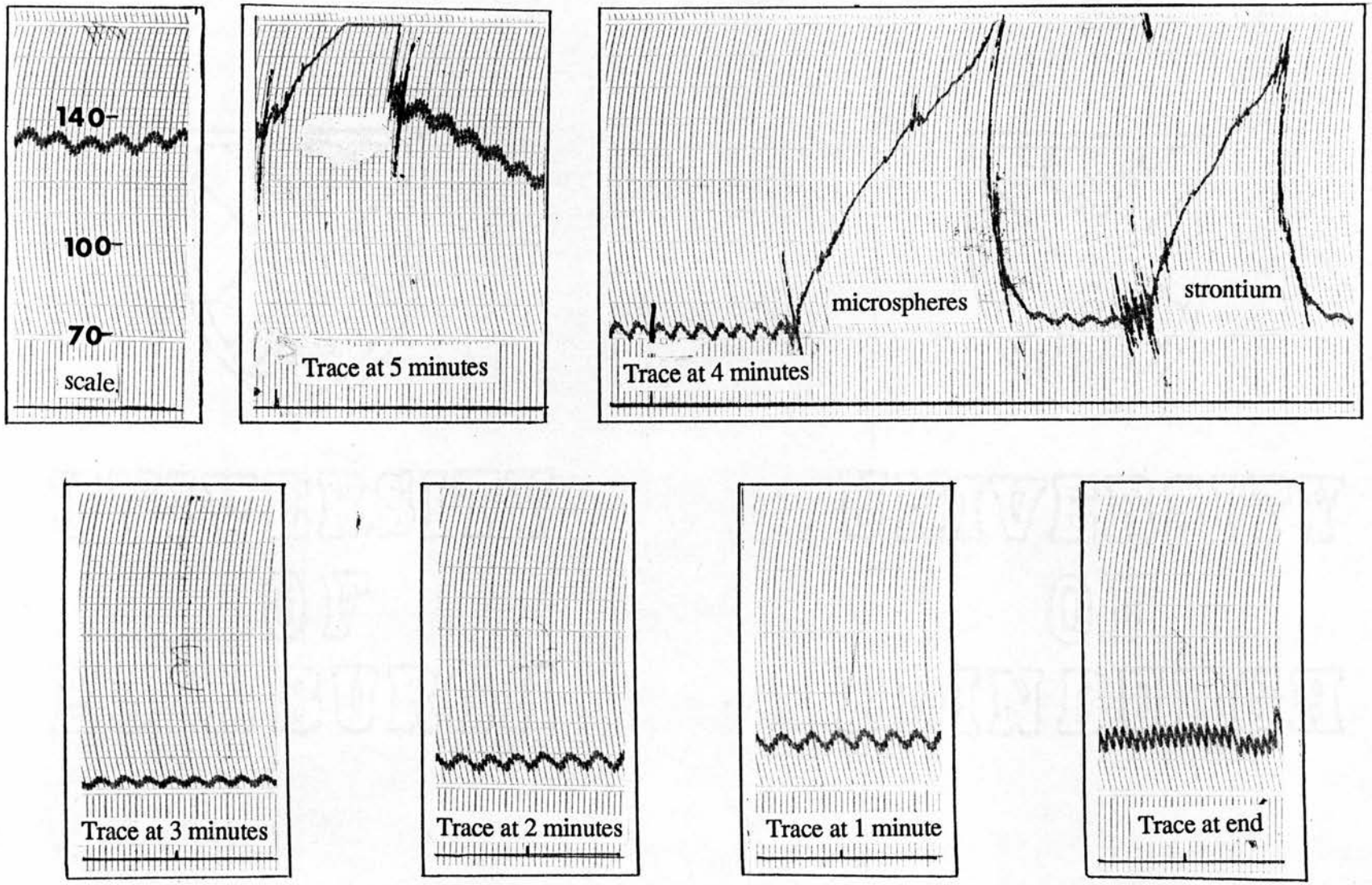


Figure 5.6. An example of the ECG. and heart rate from an animal given 12μg PTH

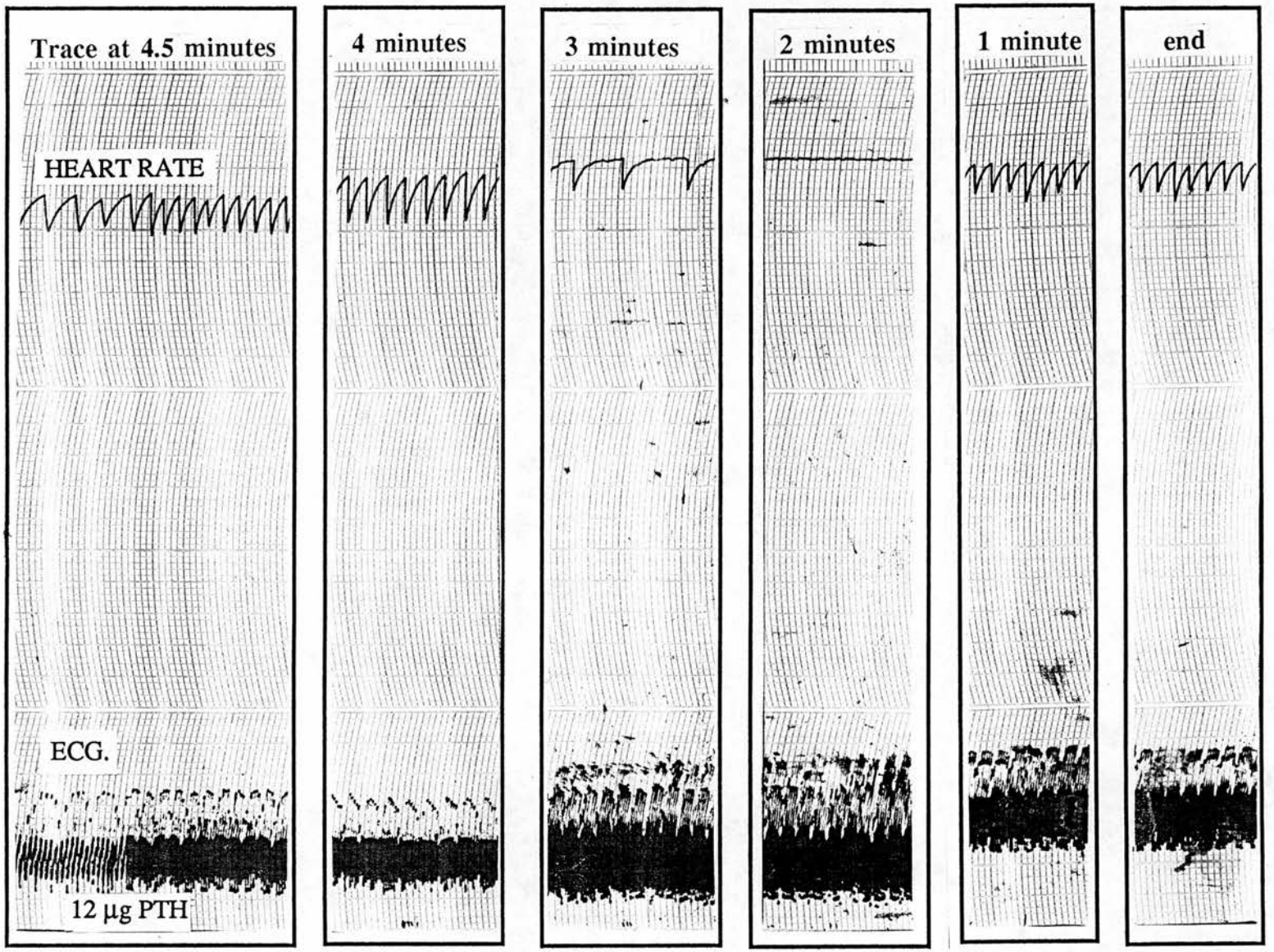
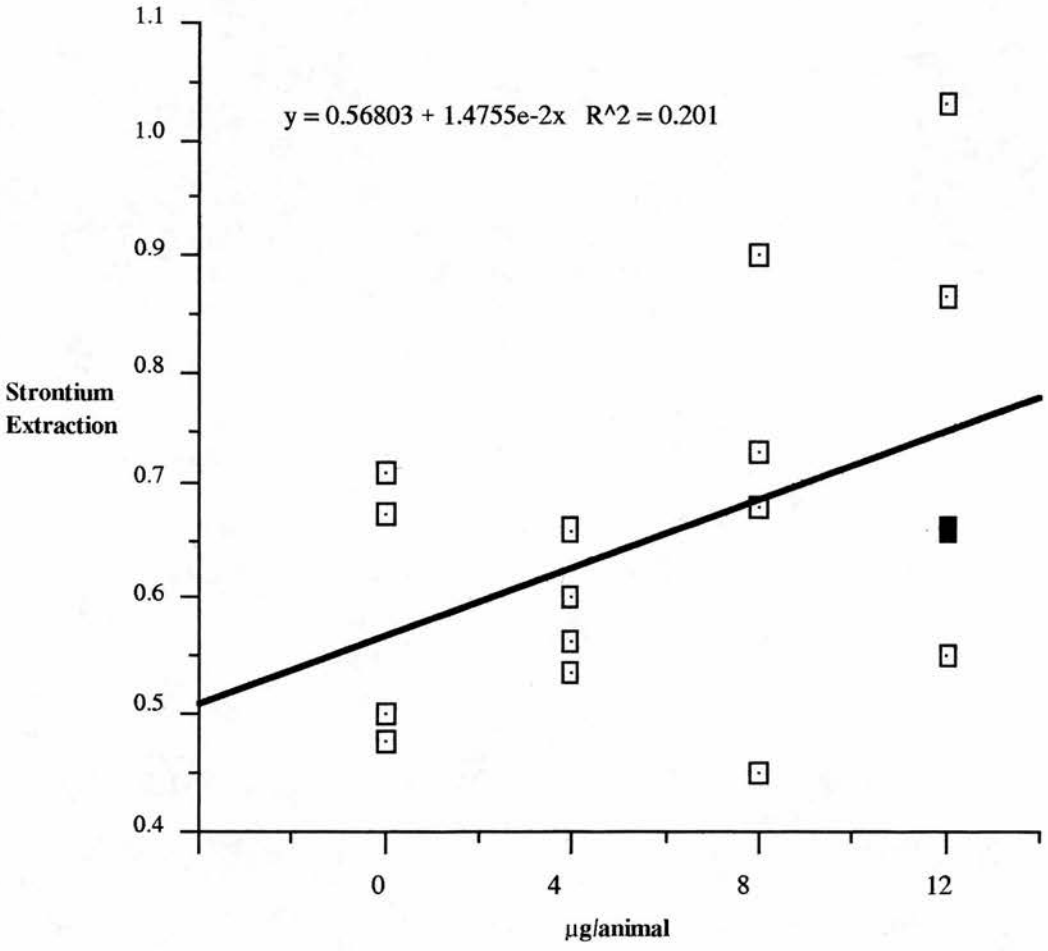


Figure 5.7. Effect of PTH on Strontium Extraction.

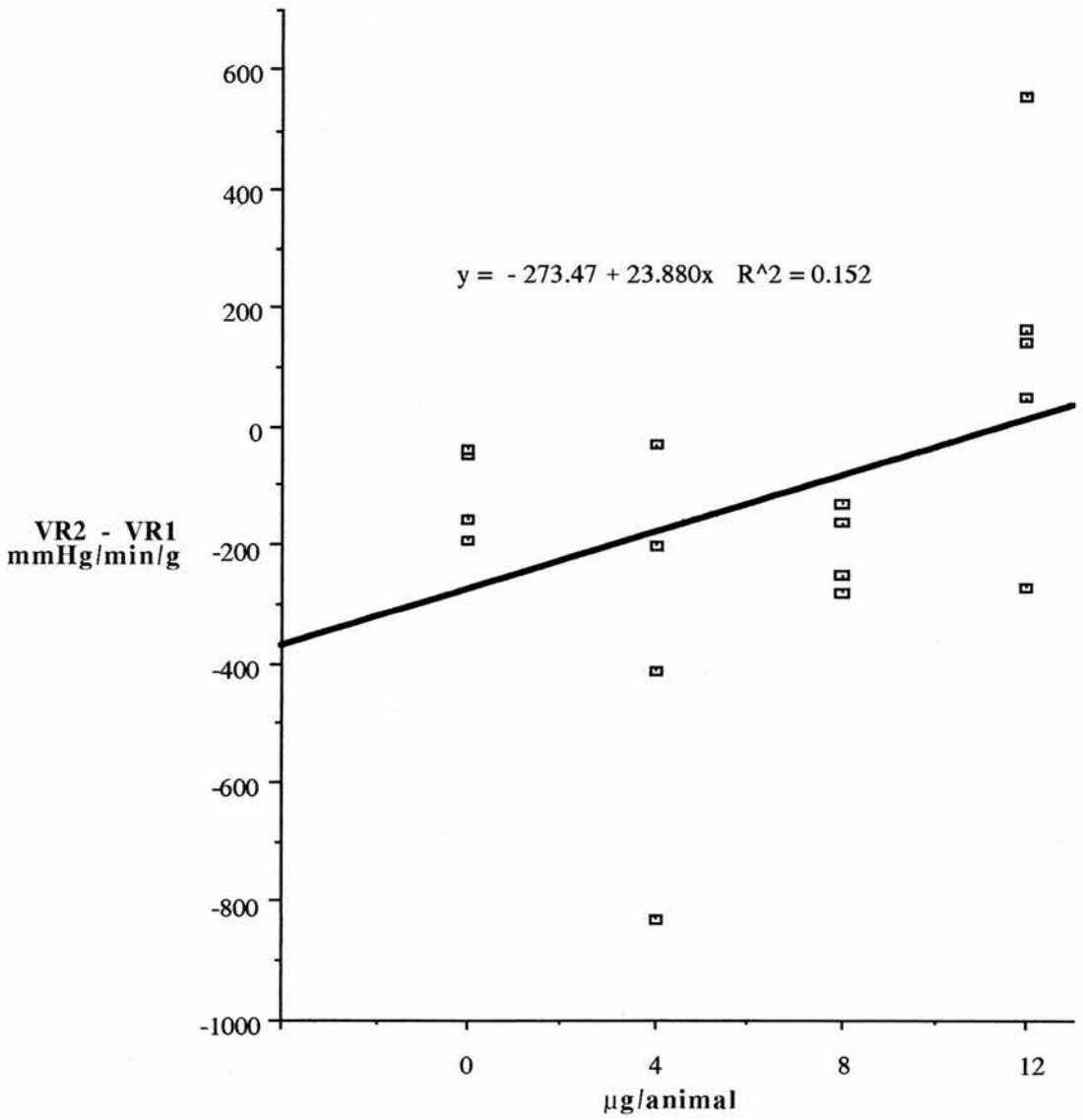


■ Two points

Significance of the regression, $p<0.07$.

Correlation coefficient (r) = 0.448.

Figure 5.8. Change in Vascular Resistance in Response to PTH

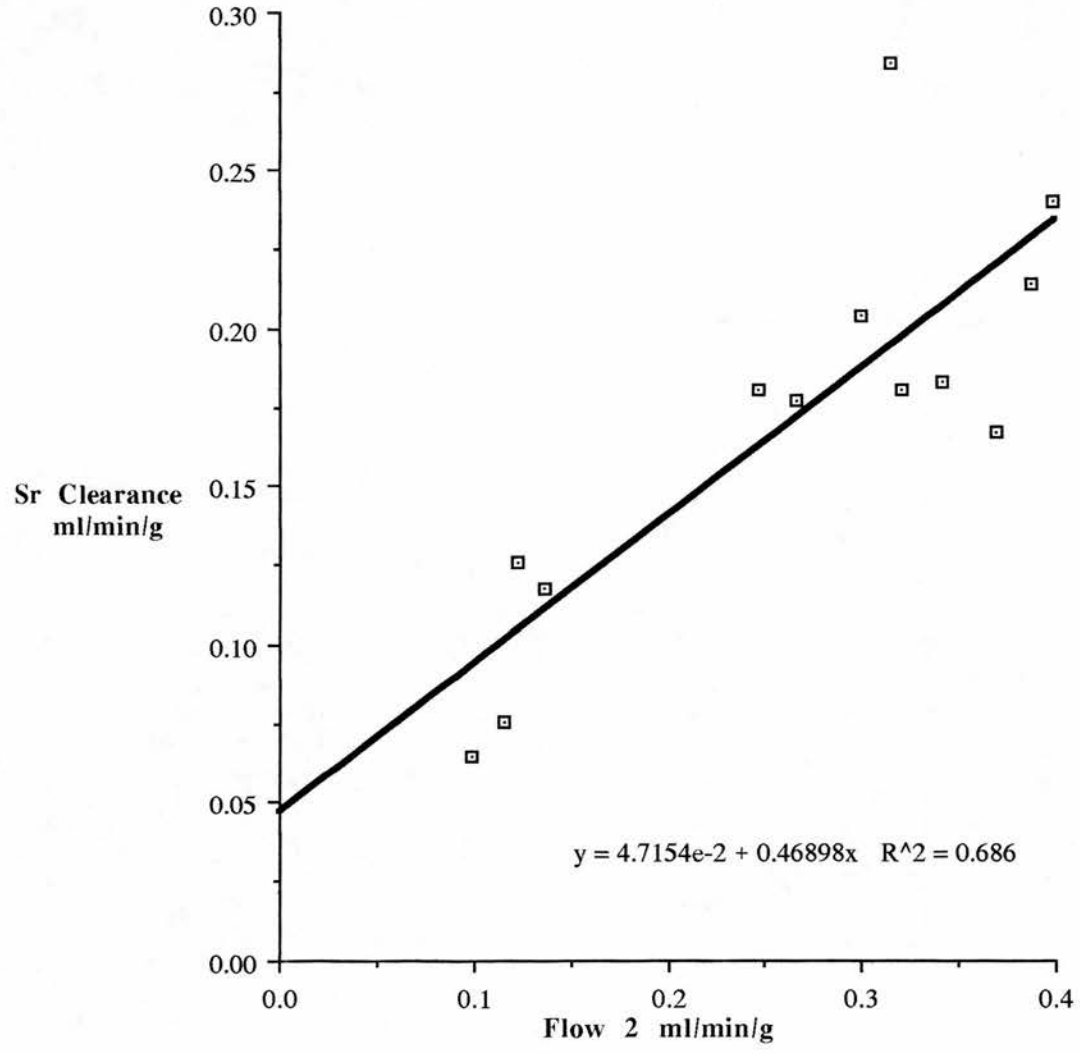


V.R.1 = Vascular Resistance as estimated from cobalt flow and blood pressure (B1.)
V.R.2 = Vascular Resistance as estimated from tin flow and blood pressure (B3).

Significance of regression, $p < 0.12$

Correlation coefficient (r) = 0.39

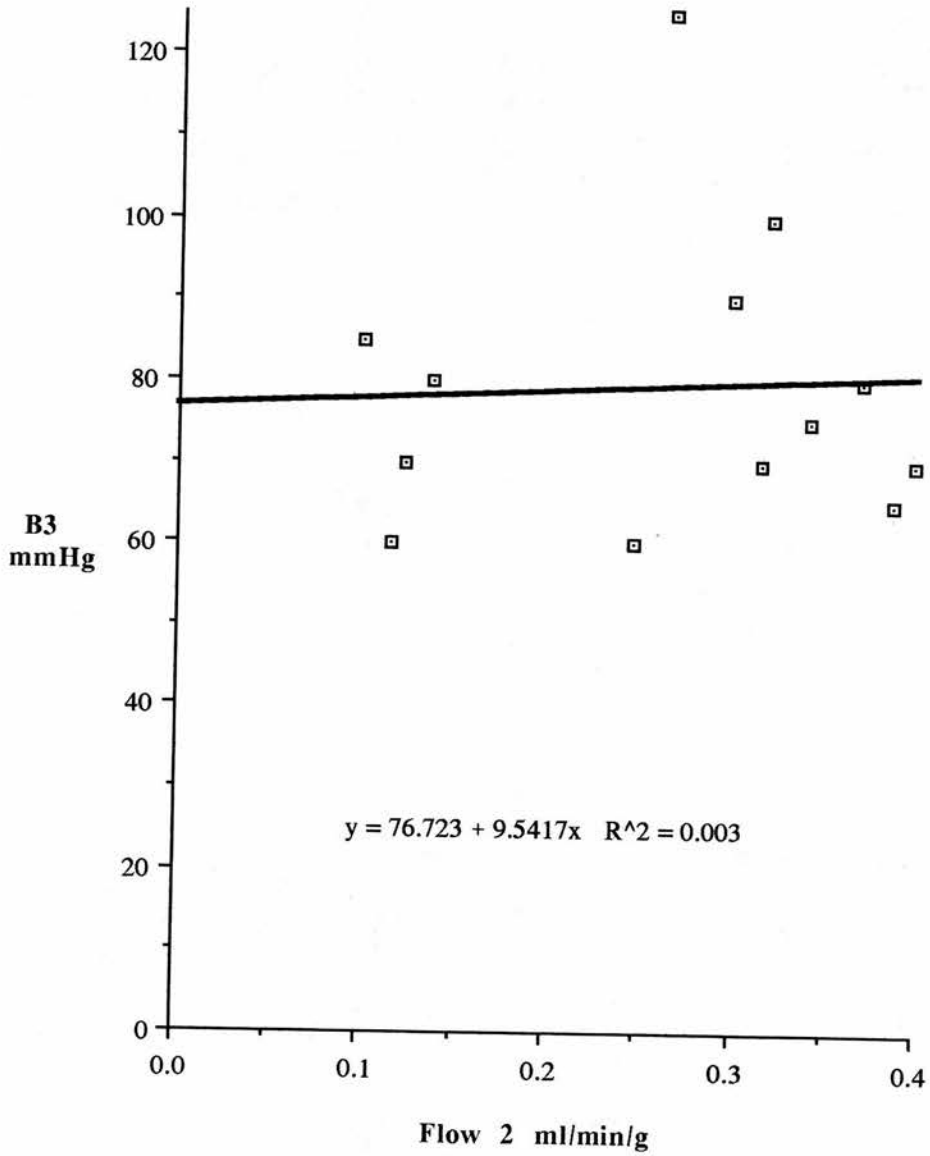
Figure 5.9. Flow Versus Strontium Clearance in the PTH Treated Animals



Significance of the regression, $p \leq 0.0005$

Correlation coefficient (r) = 0.83

Figure 5.10. Flow 2 Vesus Blood Pressure in PTH Animals



Significance of the regression, $p \leq 0.85$

Correlation coefficient (r) = 0.06

b) Prostaglandin E2

In total twenty nine animals were treated with 0, 20, 40, 60 or 80 µg prostaglandin E2. However in nine of these insufficient cobalt microspheres were collected in the blood samples (less than 300). At this time the microsphere supply was nearly finished and diluting may have meant that an insufficient number was used; also it was noticed that the microspheres tended to clump. The effects on the remaining twenty animals are summarised in tables 5.5.-5.8. These include mean values for bone blood flow, strontium clearance and change in arterial pressure (Table 5.5.), strontium extraction and bone vascular resistance (Table 5.6.) and blood flow and vascular resistance in muscle in Table 5.7. The last table gives full details of arterial blood pressure at the four points of interest (Table 5.8.).

Prostaglandin E2 produced a significant effect on bone blood flow, clearance, extraction, vascular resistance and arterial blood pressure. Treatment with this hormone resulted in a dose dependent decrease in bone blood flow (Figure 5.11.). In all cases the mean flow 2 values are statistically different from the control mean ($p<0.02$) and each is lower than their respective flow 1 values (Table 5.5.) .

i.e. 20µg Flow 2 = 71% of Flow 1

40µg Flow 2 = 55% "

60µg Flow 2 = 42% "

80µg Flow 2 = 47% "

The effect on bone blood flow is significant at 20, 40 and 80µg (Table 5.5.). The correlation between the two variables is in the region of 0.68 indicating the extent to which the two are related, with the drug responsible for some 45% of the variation, calculated from the r squared value of 0.45 (Figure 5.11.).

Similarly strontium clearance is reduced with increasing dose of PGE2 (Figure 5.12.) and this is statistically significant, $p<0.001$ (DF=20). Comparison with the control mean values shows that in each case clearance is less than that of the control (85%, 73% 65% and 65% respectively). The extent of variation that the drug is responsible for is approximately 46%, ($r^2= 0.46$) but the value for correlation i.e. -0.68 confirms that the

two are related to some extent. The mean values for clearance are statistically different from that of the control at the 40, 60 and 80µg doses (Table 5.5.).

Prostaglandin E2 treatment also decreases arterial pressure in a dose dependent fashion (Figure 5.13.). The mean percentage decrease ranges from 47% at 20µg to 43% at 80µg indicating that the initial effect is similar at all doses. However it is the duration of this arterial pressure decrease that varies with increasing dose. Like the other agents used in this study the time taken to recover increases with increasing dose (Figure 5.14.).

Both vascular resistance and strontium extraction increase significantly (the line of regression is significantly different from zero) in a dose dependent fashion (Figures 5.14 and 5.16.). A change in vascular resistance can be anticipated as PGE2 acts on bone reducing flow. Vascular resistance is calculated by dividing bone blood flow by blood pressure thus this increase infers that flow is decreased to a greater degree than blood pressure. A plot of these two variables confirms this to be the case (Figure 5.18).

The increase in extraction can be inferred from the decrease in clearance however this increase need not be significant as extraction is also dependent on flow, which in this case also decreases. However the change in extraction is significant (Figure 5.14.) thus the decrease in clearance and flow cannot be of the same degree. This is demonstrated in figures 11 and 12, the slope (b) is steeper for change in flow than strontium clearance. Correlation and regression analysis of flow versus strontium clearance in the 20 - 80µg animals shows that the two variables are highly correlated ($r=0.76$), 59% of the variation found in clearance resulting from a variation in flow (Figure 5.17.). The significance of the regression is 0.0008 with a slope of 0.78 (s.e. 0.096). Comparing this value with that of the control data results in a significant t-value ($p<0.01$). This suggests that PGE2 treatment affects clearance in some way independent of its action on bone blood flow.

In muscle the flow is not significantly affected by PGE2 treatment, although, like PTH, this does change, but the standard deviation of the means are high and at no dose are the changes significant (Table 8). Similarly the change in muscle vascular resistance at any one dose is not significant and neither is the regression analysis.

Table 5.5. Effect of prostaglandin E2 on blood flow, strontium clearance and blood pressure in rat bone

		PGE2 µg/animal			
	Control	20	40	60	80
Flow					
Cobalt	0.49 ± 0.11	0.32 ± 0.04	0.31 ± 0.13	0.33 ± 0.14	0.36 ± 0.06
Tin	0.50 ± 0.1	0.23 ± 0.03	0.17 ± 0.05	0.14 + 0.03	0.17 ± 0.03
@Sn - Co	0.01 ± 0.07	-0.09 ± 0.05	-0.14 ± 0.09	-0.20 ± 0.14	-0.19 ± 0.06
***Sr Clear**	0.32 ± 0.02	0.28± 0.06	0.25 ± 0.04	0.22 ± 0.04	0.22 ± 0.04
Blood Pressure					
(B4 - B2)	5 ± 7	-57± 40	-52 ± 3	-77 ± 17	-55 ± 4

@ Paired t-test --- 20, 40µg $p \leq 0.05$
80µg $p \leq 0.01$

* Unpaired t-test (comparison with 0 dose) --- 40µg $p \leq 0.05$
60, 80µg $p \leq 0.01$

All values represent mean ± standard deviation.

Number of animals in each group (n) = 4.

Flow is measured in ml/min/g.

Blood pressure is measured in mmHg/min/g ; at two points.

(B2) at begining of experimental run,

(B4) at point of maximum effect.

Table 5.6. Effect of prostaglandin E2 on strontium extraction and vascular resistance in rat bone

		PGE2 µg/animal			
	Control	20	40	60	80
***Strontium Extraction**					
	0.66 ± 0.11	1.23 ± 0.18	1.6 ± 0.29	1.6 ± 0.19	1.36 ± 0.33
Vascular Resistance					
Cobalt	249 ± 46	401 ± 21	462 ± 200	456 ± 109	365 ± 57
Tin	256 ± 56	366 ± 64	471 ± 111	614 ± 134	566 ± 230
@Sn - Co	7 ± 26	-35 ± 48	8 ± 198	157 ± 108	201 ± 177

@ Paired t-test --- All N.S.

* Unpaired t-test (comparison with 0 dose) --- All $p \leq 0.002$

All values represent mean ± standard deviation.

Number of animals in each group (n) = 4.

Vascular resistance is measured in mmHg/min/g (using B1 for calculating the cobalt value and B3 the tin value).

Table 5.7. Effect of prostaglandin E2 on blood flow and vascular resistance in rat muscle

		PGE2 µg/animal			
	Control	20	40	60	80+
Flow					
Cobalt	0.21 ± 0.20	0.11 ± 0.02	0.10 ± 0.08	0.22 ± 0.11	0.21 ± 0.08
Tin	0.20 ± 0.24	0.13 ± 0.02	0.19 ± 0.06	0.20 ± 0.15	0.13 ± 0.03
@Sn - Co	-0.01 ± 0.05	0.02 ± 0.03	0.08 ± 0.12	-0.03 ± 0.08	-0.08 ± 0.10
Vascular Resistance					
Cobalt	881 ± 530	1187 ± 178	1824 ± 1280	750 ± 353	671 ± 324
Tin	1132 ± 626	663 ± 104	435 ± 157	541 ± 240	736 ± 246
@Sn - Co	252 ± 277	-523 ± 249	-1388 ± 1361	-209 ± 293	-65 ± 495

@ Paired t-test --- All N.S.

All values represent mean ± standard deviation.

Number of animals in each group (n) = 4.

\+ (n) = 3.

Vascular resistance is measured in mmHg/min/g (using B1 for calculating the cobalt value and B3 the tin value)

Table 5.8. Mean and standard deviation of blood pressure measured at the four reference points for prostaglandin E2 animals

			PGE2 µg/animal			
		Control	20	40	60	80
Blood pressure						
Cobalt	B1	120 ± 25	126 ± 14	122 ± 10	141 ± 13	129 ± 9
Tin	B2	119 ± 24	129 ± 16	120 ± 8	149 ± 13	125 ± 4
	B3	124 ± 18	82 ± 9	75 ± 13	81 ± 8	90 ± 21
	B4	126 ± 18	62 ± 10	68 ± 6	71 ± 10	70 ± 4

@ Paired t-test --- All N.S.

All values represent mean ± standard deviation.

Number of animals in each group (n) = 4.

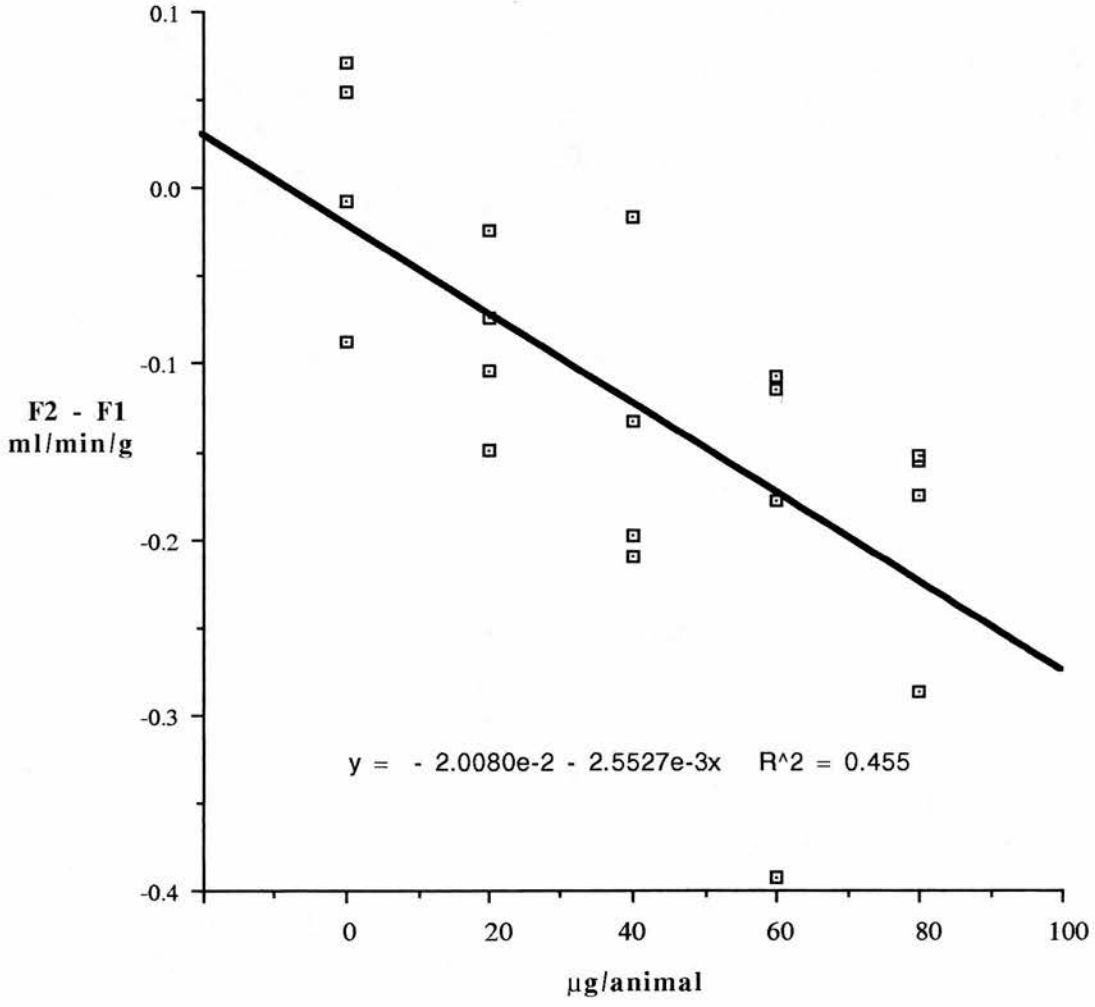
Blood pressure is measured in mmHg.

B1 and B3 Blood pressure at point of microsphere injection used for calculation of vascular resistance.

B2 Blood pressure at the begining of the experimental run, used for the calculation of blood pressure change (B4 - B2).

B4 Blood pressure at the point of maximum drug effect.

Figure 5.11. Change in Bone Blood Flow After Injection of PGE2.

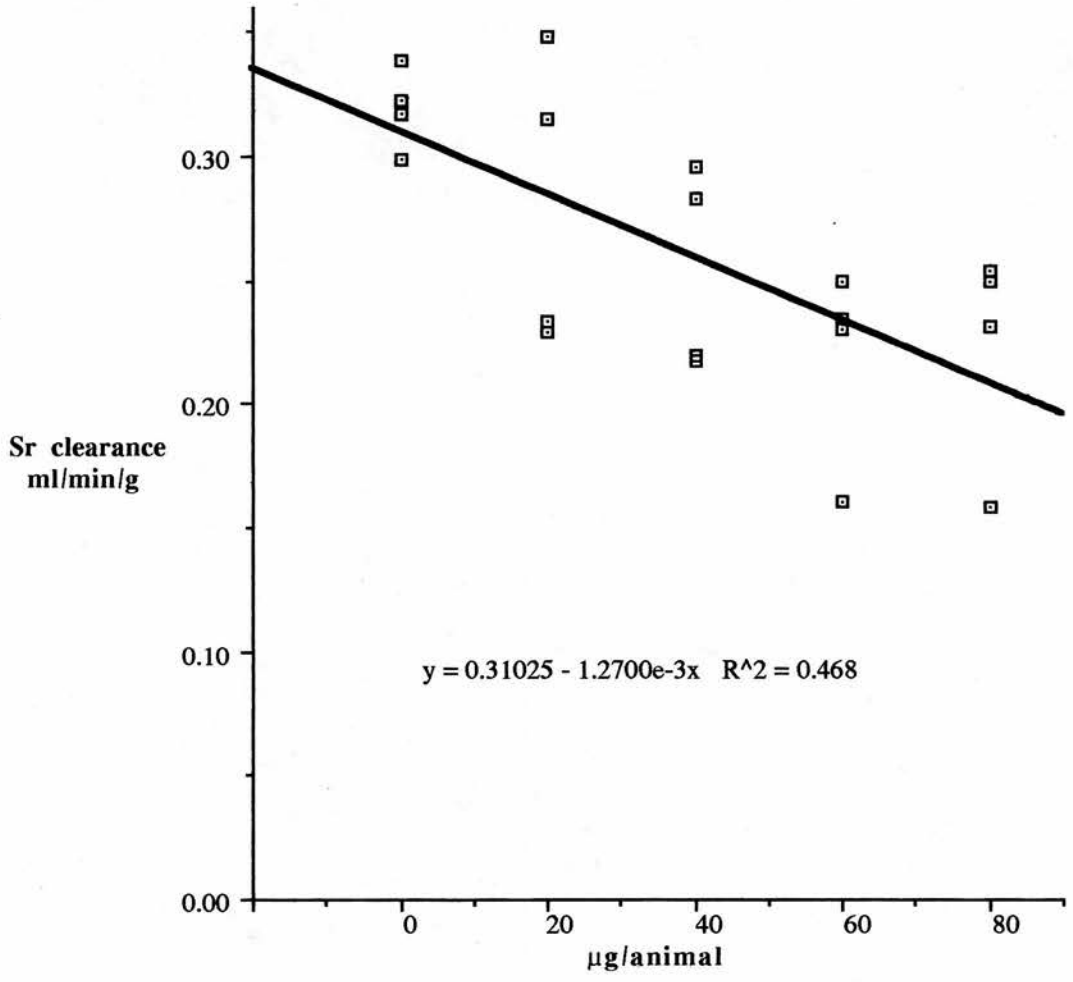


F1 = Control Blood Flow -- using Co microspheres.
F2 = Experimental Blood Flow --- using tin microspheres.

Significance of regression , $p<0.001$

Correlation coefficient (r) = - 0.67

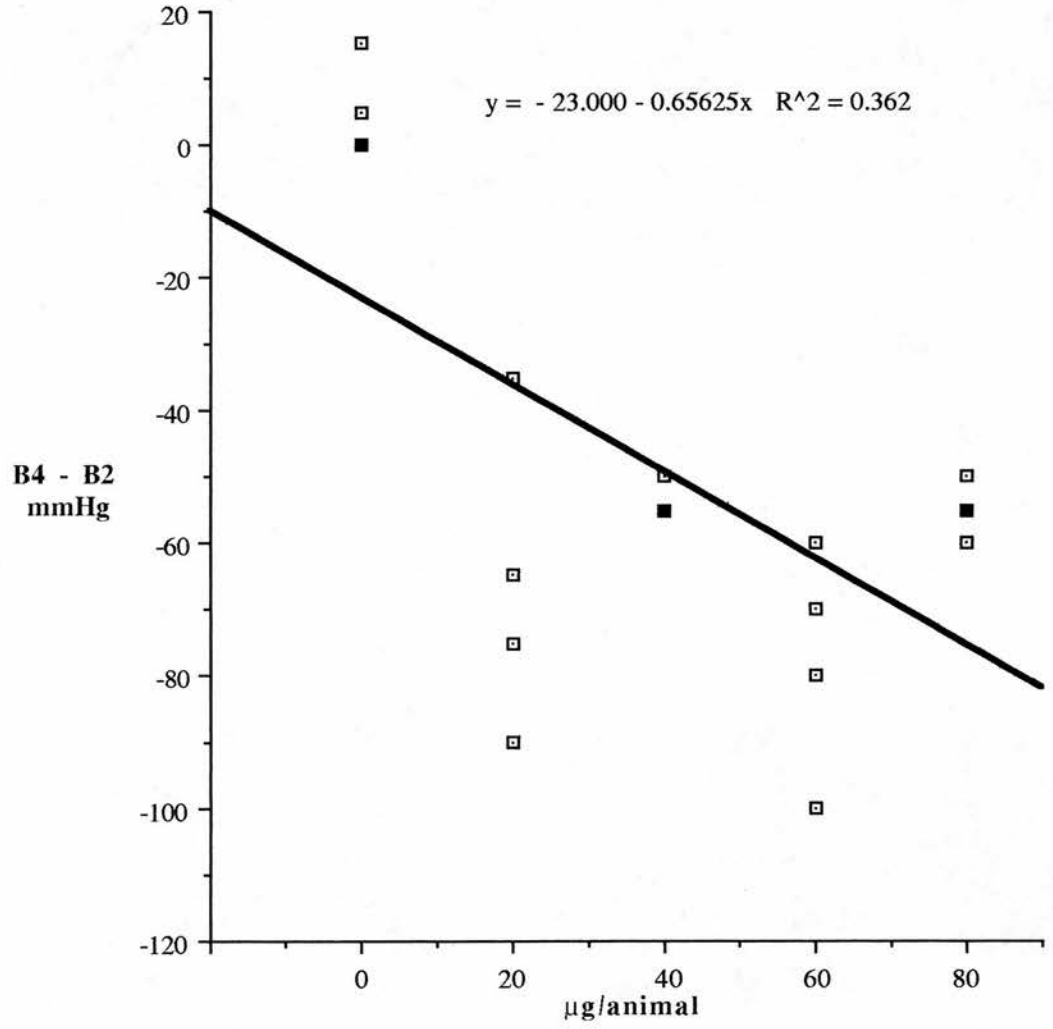
Figure 5.12. Effect of PGE2 Treatment on Strontium Clearance



Significance of the regression, $p \leq 0.001$

Correlation coefficient (r) = - 0.68.

Figure 5.13 Change in Blood Pressure With PGE2 Treatment



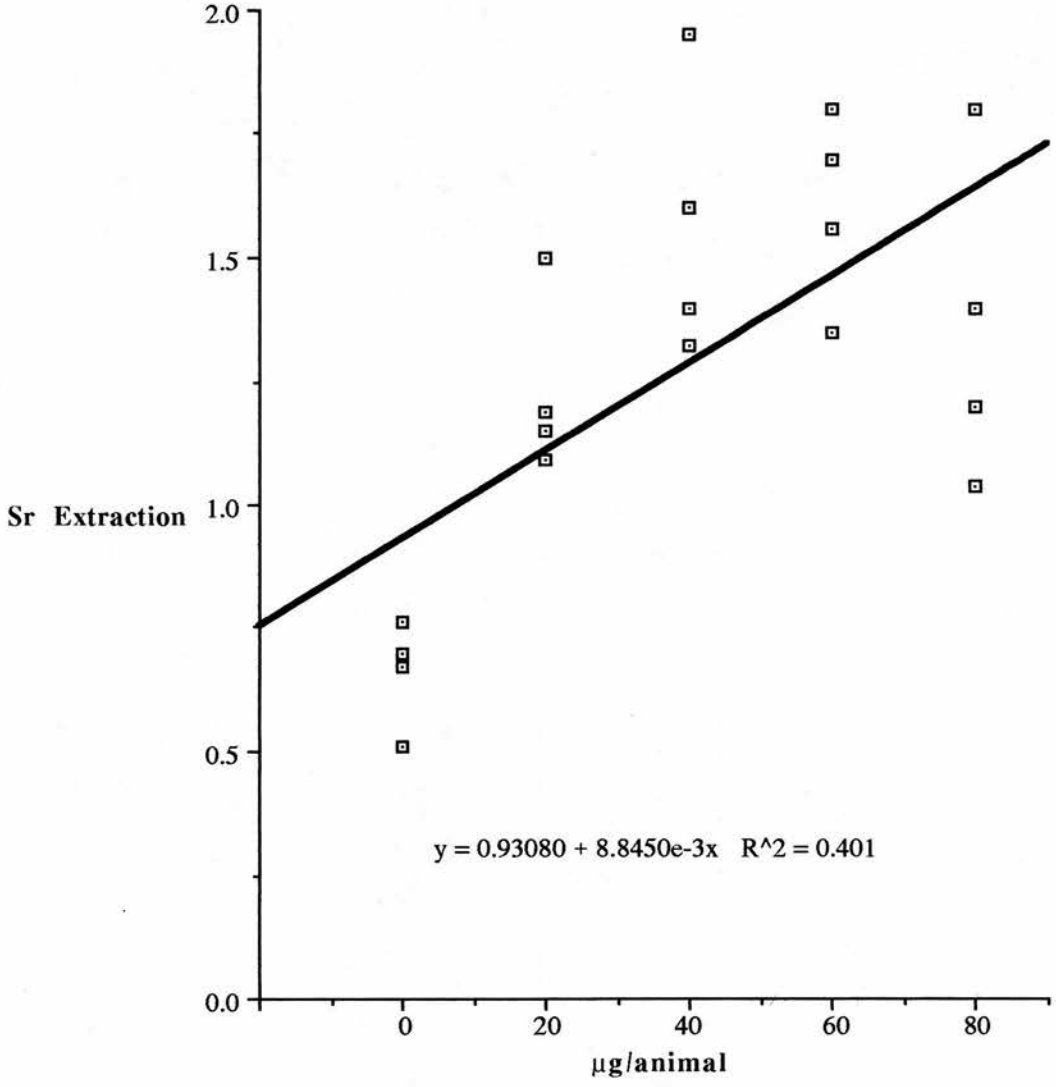
■ Two points

B2 = Arterial Pressure measured at begining of experimental period .
B4 = Arterial Pressure measured at point of maximum drug effect.

Significance of the regression, $p < 0.005$

Correlation coefficient (r) = - 0.6

Figure 5.14. Effect of PGE2 on Strontium Extraction



Significance of the regression, $p<0.003$

Correlation coefficient (r) = 0.633 .

Figure 5.13. An example of the arterial pressure trace from an animal given 80 μg PGE2

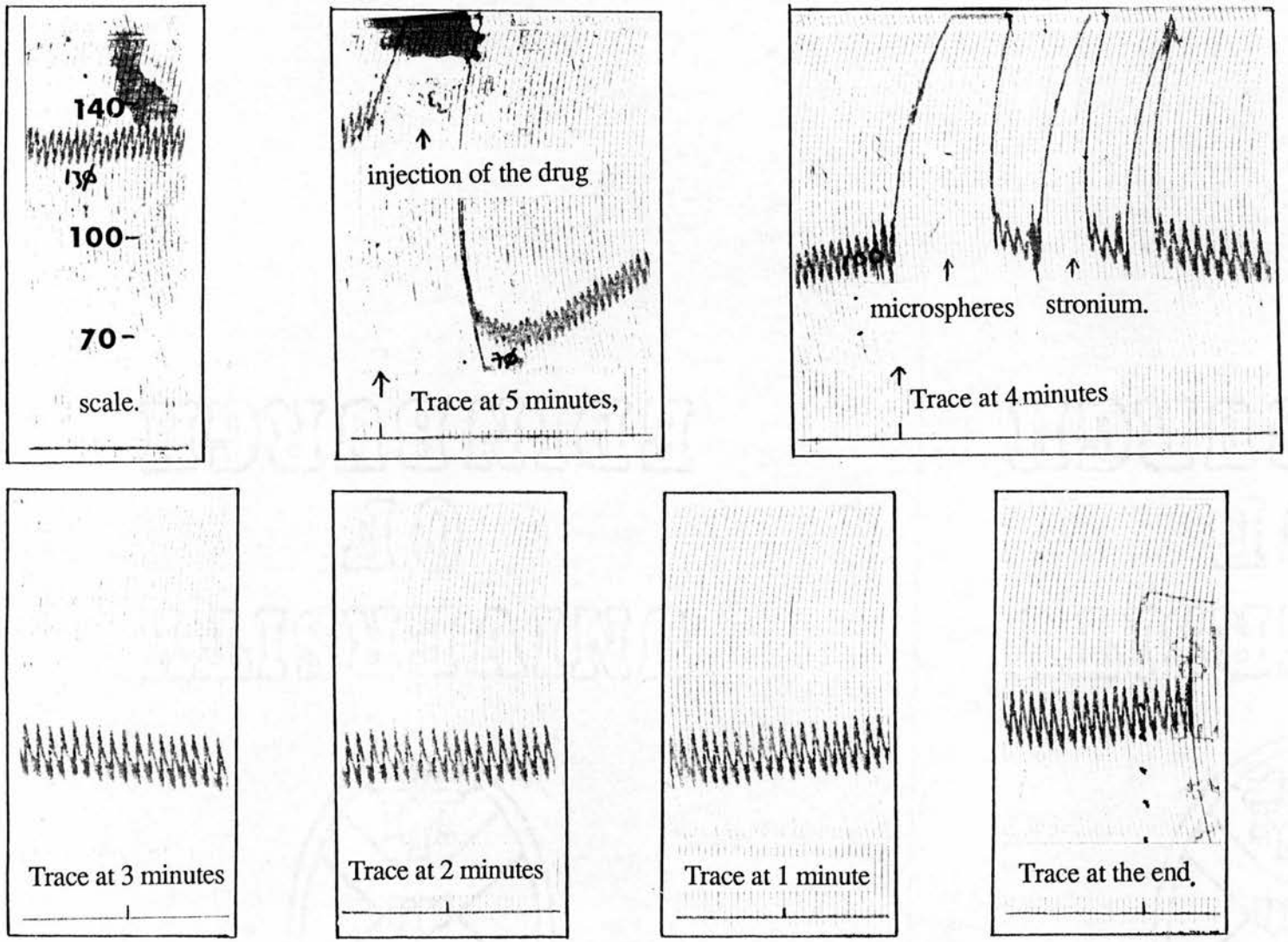
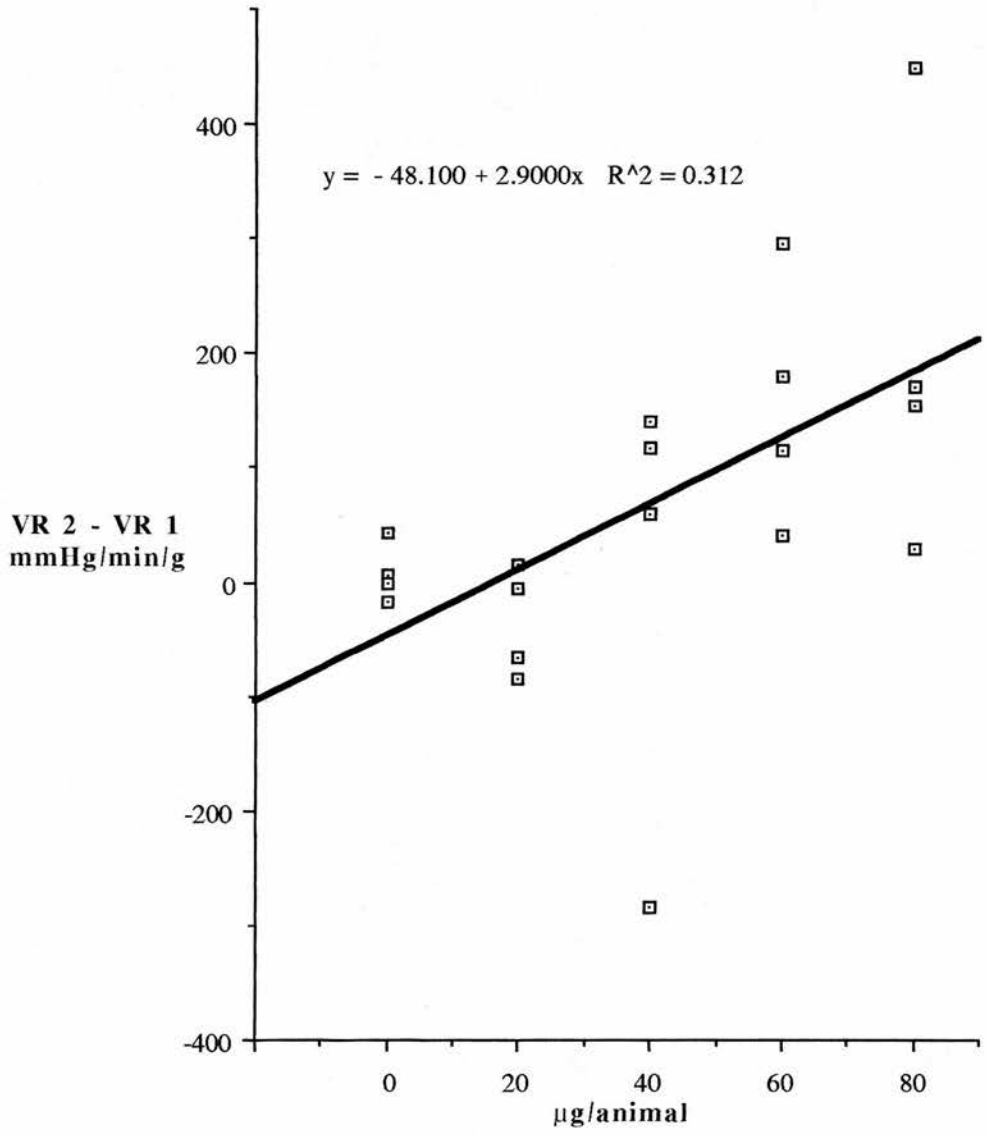


Figure 5.16. Change in Bone Vascular Resistance With PGE2 Treatment

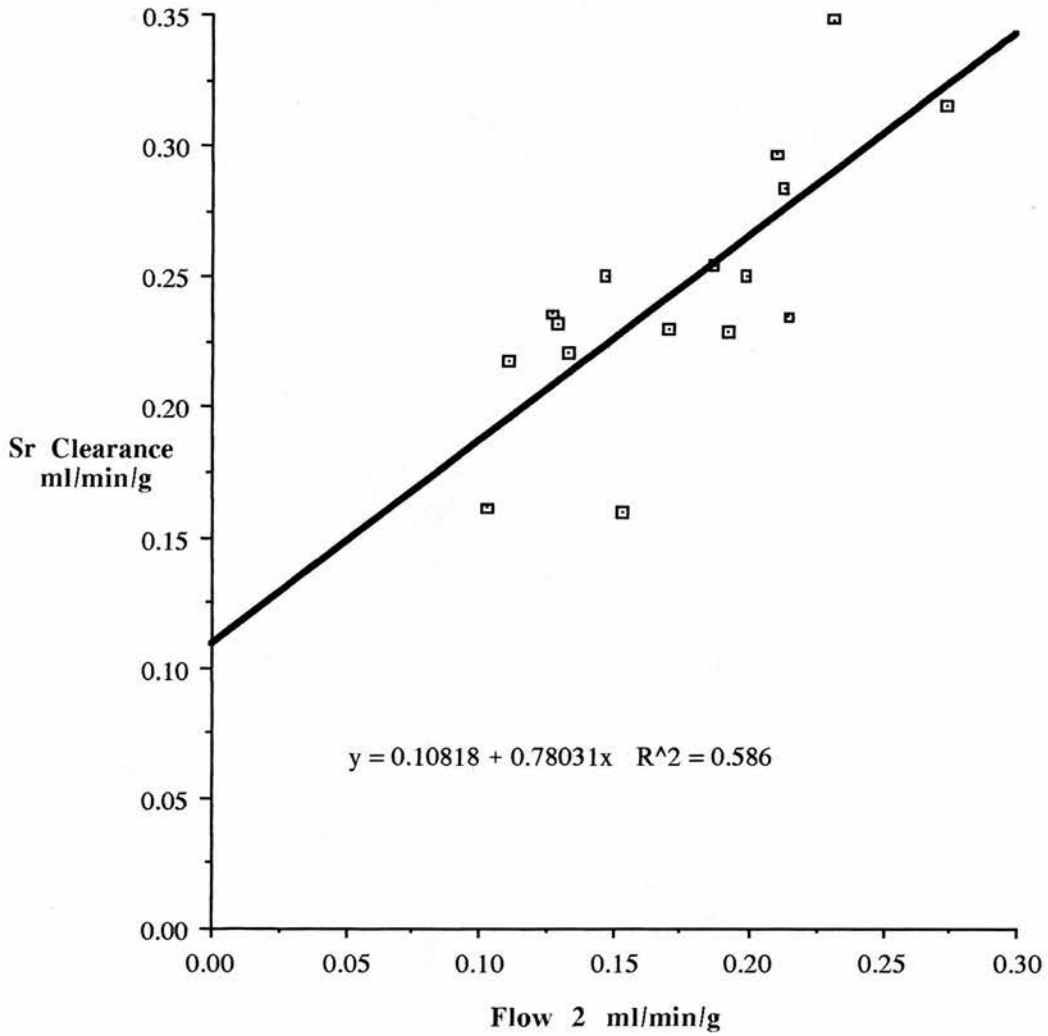


V.R.1 = Vascular Resistance as estimated from cobalt flow and blood pressure (B1.)
V.R.2 = Vascular Resistance as estimated from tin flow and blood pressure (B3).

Significance of the regression, $p \leq 0.01$.

Correlation coefficient (r) = 0.56

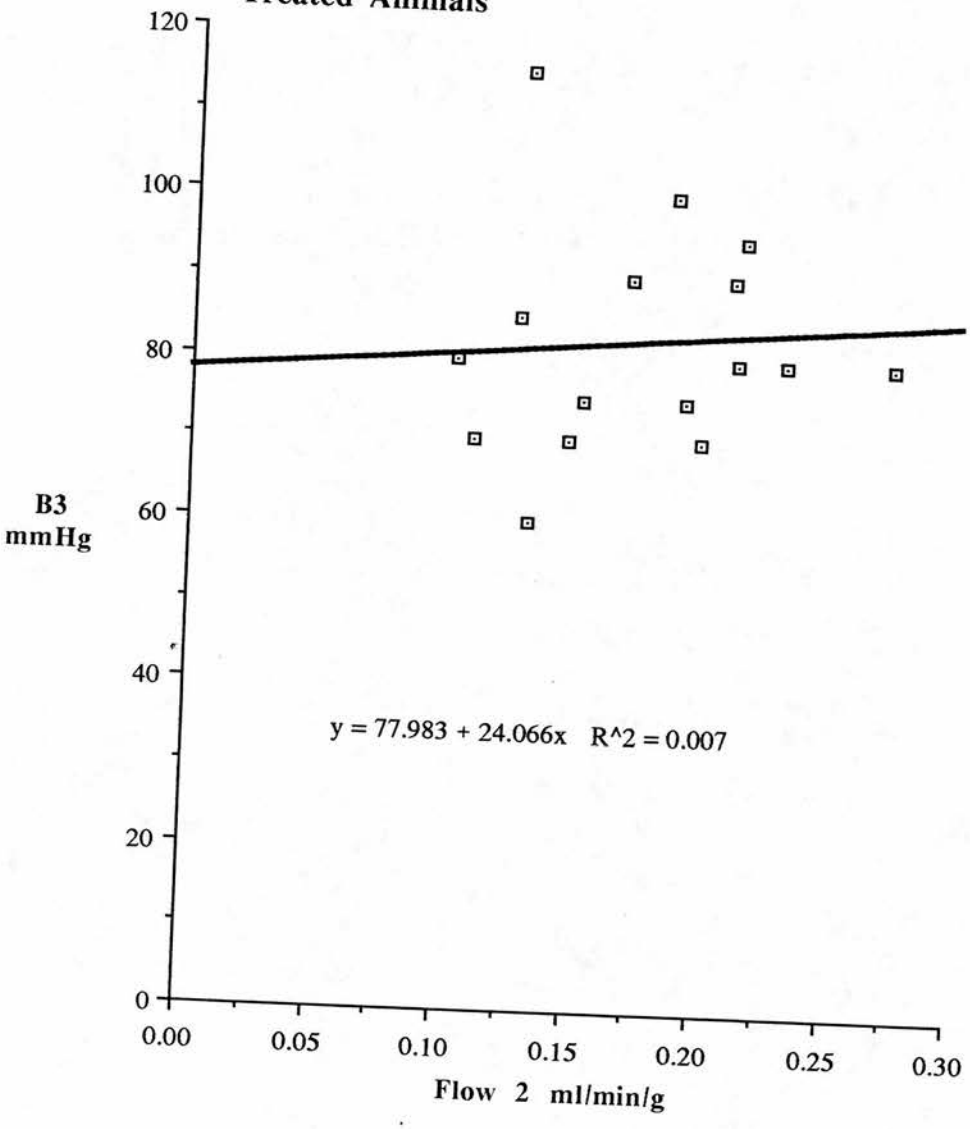
Figure 5.17. Flow Versus Strontium Clearance in PGE2 Treated Animals



Significance of the regression, $p \leq 0.0005$

Correlation coefficient (r) = 0.77

Figure 5.18. Flow Versus Blood Pressure in the PGE2 Treated Animals



Significance of the regression, $p \leq 0.75$

Correlation coefficient (r) = 0.09

c) Calcitonin

Twenty eight animals were treated with 0, 10, 25, or 50 units of Salmon calcitonin. Seven rats were unsuitable for inclusion in this study, in four there as insufficient microspheres in the blood sample or the sample was of an inadequate volume, two animals died of anaesthetic death and in one there was a question as to the actual dose received.

The effect of calcitonin on bone blood flow, strontium clearance and arterial pressure in the remaining 21 animals is detailed in Table 5.9. While Table 5.10 includes the mean values for strontium extraction and bone vascular resistance and Table 5.11. the blood flow and vascular resistance in muscle. Finally the arterial pressure values are detailed in Table 5.12.

The only significant action of calcitonin is upon strontium clearance ($p<0.03$ Figure 5.20a.). However it is only the change at fifty units that causes this effect. At the other doses a the line of regression if plotted is almost horizontal with a p value of 0.8 (Figure 5.20b.). This suggests that a higher dose could have been included in the study to see if the change continues with increased dose. Possibly the dose response curve for calcitonin is initially flat and then begins to curve upwards. The first two doses may be on this relatively flat portion with the larger dose on the steeper portion. However the 10U. dose was chosen because Driessens et al (1979) found this, and lower doses had a significant effect. This study however was in the perfused dog tibia and the rat does not necessarily exhibit the same dose response.

It is the fifty unit dose that affects the slope of the lines of regression for flow and vascular resistance change. Initially the flow decreases at 10U flow 2 is 80% of flow 1, at 25U this falls to 70% but the increase of 42% in the 50U dose tends to balance this out and the regression line is not significant (Figure 5.19.). Vascular resistance exhibits the opposite pattern initially increasing with decrease shown at the 50U. dose (Figure 5.24.). It is at fifty units that the only significant t-values are found in both flow change and strontium clearance.

Strontium extraction is dependent upon flow and clearance, a significant change in either does not necessarily affect it, as is the case here (Figure 5.22). That is strontium clearance decreases with increasing dose of calcitonin but this is not significant.

Calcitonin has no effect on blood pressure with a mean and standard deviation during the experimental flow of 121 ± 14 (n=4) for the control and 134 ± 18 (n=14) for the treated animals (Figure 5.21.) Within each animal the pressure remained constant during the test period i.e. B2, B3 and B4 are all the same, indicating that the drug has no effect on the vascular network (Table 5.11.). Similarly there is no significant effect on either flow or vascular resistance in rat muscle over any of these doses (Table 5.11.).

Analysis of flow versus strontium clearance in the 10-50U. animals is presented in figure 2 7. There is a correlation between the two variables but this is lower than that of any other group. Comparison of the slope with that of the control group, PTH experimental and the PGE2 experimental produces a non significant t value in each case. Thus this appears to represent a standard effect, that is the relationship is demonstrated in this range in all animals regardless of drug treatment.

Table 5.9. **Effect of calcitonin on blood flow, strontium clearance and blood pressure in rat bone**

		Calcitonin U/animal		
	Control#	10+	25 #	50 #
Flow				
Cobalt	0.29 ± 0.06	0.30 ± 0.03	0.45 ± 0.11	0.29 ± 0.11
Tin	0.28 ± 0.07	0.24 ± 0.07	0.32 ± 0.12	0.41 ± 0.12
@Sn - Co	-0.01 ± 0.08	0.06 ± 0.08	0.13 ± 0.11	0.12 ± 0.11
*** Sr clearance**				
	0.20 ± 0.02	0.20 ± 0.04	0.19 ± 0.05	0.26 ± 0.06
Blood Pressure Change				
	!	#	!	#
(B4 - B2)	1 ± 2.5	0	0	1 ± 2.2

@ Paired t-test --- All N.S.

* Unpaired t-test (comparison with 0 dose) --- 50 U. $p \leq 0.05$

All values represent mean ± standard deviation.

Number of animals in each group ! (n) = 4.

(n) = 5.

+ (n) = 6.

Flow and clearance are measured in ml/min/g.

Blood pressure is measured in mmHg; at two points;

(B2) at the begining of the experimetal run,

(B4) at point of maximum effect.

Sn = Tin. Co = Cobalt.

Table 5.10. Effect of calcitonin hormone on strontium extraction and vascular resistance in rat bone

		Calcitonin U./animal		
	Control+	10#	25+	50+
***Strontium Extraction**				
	0.72 ± 0.1	0.87 ± 0.23	0.68 ± 0.26	0.65 ± 0.16
Vascular Resistance				
Cobalt	461 ± 130	445 ± 37	339 ± 59	548 ± 213
Tin	424 ± 108	592 ± 215	526 ± 229	343 ± 85
@Sn - Co	-37 ± 77	152 ± 179	191 ± 189	216 ± 240

@ Paired t-test --- All N.S.

* Unpaired t-test (comparison with 0 dose) --- All N.S.

All values represent mean ± standard deviation.

Number of animals in each group + (n) = 5

\# (n) = 6

Vascular resistance is measured in mmHg/min/g (using B1 for calculating the cobalt value ad B3 the tin value).

Sn = Tin. Co = Cobalt.

Table 5.11. Effect of calcitonin infusion on blood flow and vascular resistance in rat muscle

	Control	10+	25#	50!
Flow				
Cobalt	0.08 ± 0.03	0.11 ± 0.05	0.08 ± 0.01	0.12 ± 0.02
Tin	0.08 ± 0.13	0.11 ± 0.05	0.08 ± 0.03	0.15 ± 0.07
@Sn - Co	-0.001 ± 0.04	-0.02 ± 0.06	0.001± 0.003	-0.02 ± 0.09
Vascular Resistance				
	!	!	#	!
Cobalt	1520 ± 491	1736 ± 1275	2095 ± 446	1192 ± 288
Tin	1492 ± 107	1953 ± 1840	2235 ± 1547	1047 ± 400
@Sn - Co	329 ± 300	253 ± 546	140 ± 1206	-135 ± 600

All values represent mean + standard deviation.
Number of animals ! (n) = 4.
(n) = 5.
+ (n) = 6.
Flow is measured in ml/min/g.
Vascular resistance is measured in mmHg/min/g. (using B1 for calculation of cobalt vascular resistance and B3 for tin vascular resistance).

@ Paired t-test --- All N.S.

Table 5.12. Mean and standard deviation of blood pressure measured at the four reference points in calcitonin treated animals

		Calcitonin U./animal			
	Control	10#	25+	50#
Blood pressure					
Cobalt B1	122 ± 13	135 ± 25	151 ± 17	141 ± 9
Tin B2}				
* B3}	121 ± 14	135 ± 17	134 ± 30	135 ± 3
B4}				

Number of animals in each group (n) = 4.

Blood pressure is measured in mmHg.

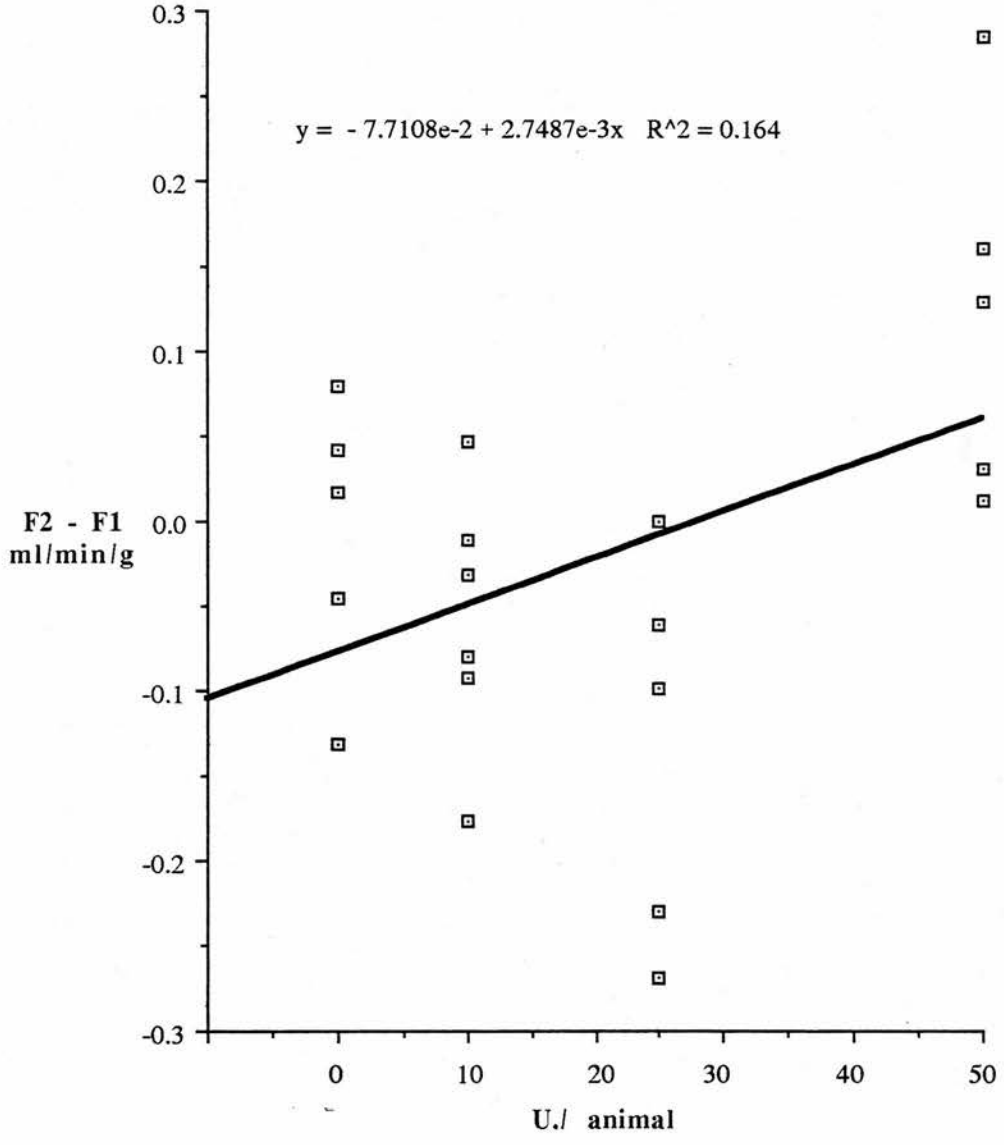
B1 and B3 Blood pressure at point of microsphere injection used for calculation of vascular resistance.

B2 Blood pressure at the begining of the experimental run, used for the calculation of blood pressure change (B4 - B2).

B4 blood pressure at the point of maximum drug effect.

* Blood pressure remains constant during infusion thus B2, B3 and B4 measurements are the same.

Figure 5.19. Change in Blood Flow During Infusion of Calcitonin

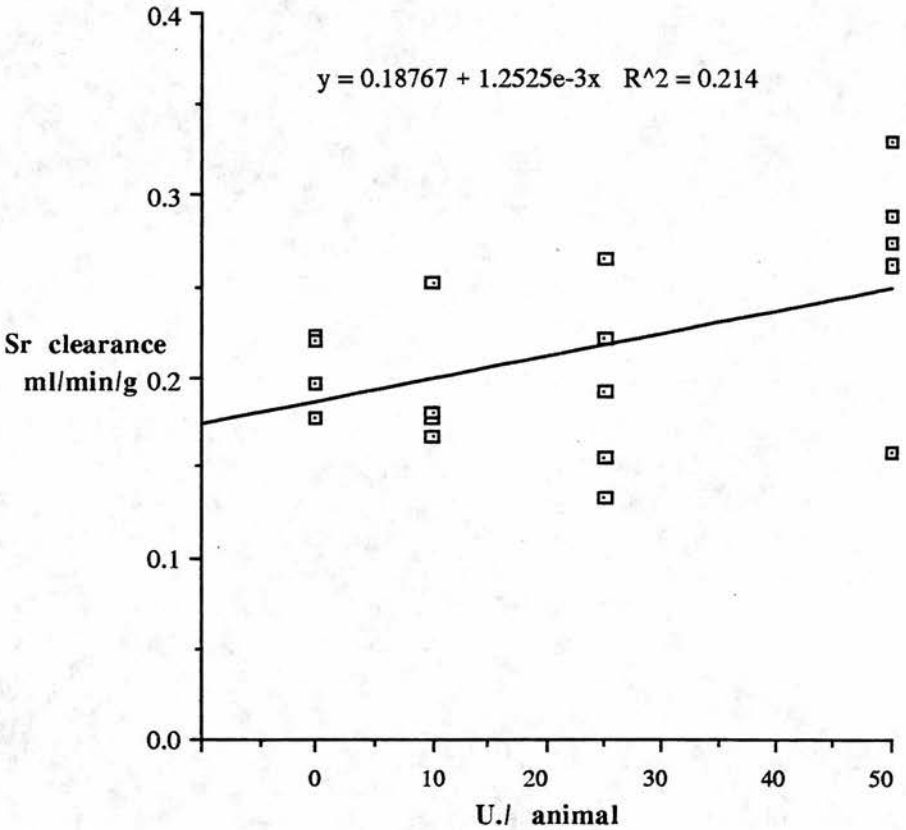


F1 = Control Blood Flow -- using Co microspheres.
F2 = Experimental Blood Flow --- using tin microspheres.

Significance of the regression , $p<0.07$.

Correlation coefficient (r) = 0.405.

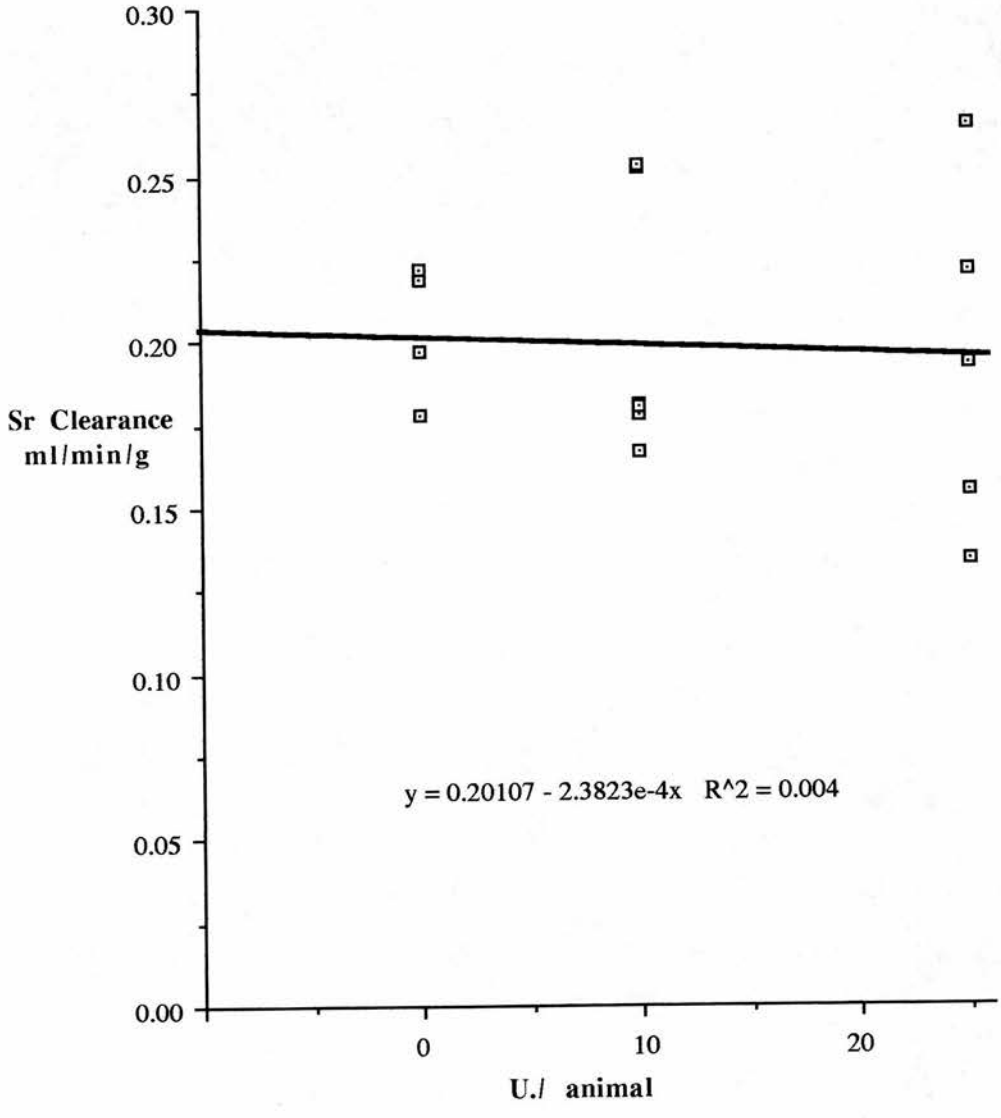
Figure 5.20a. Effect of Calcitonin on Strontium Clearance



Significance of the regression, $p<0.034$.

Correlation coefficient (r) = 0.46.

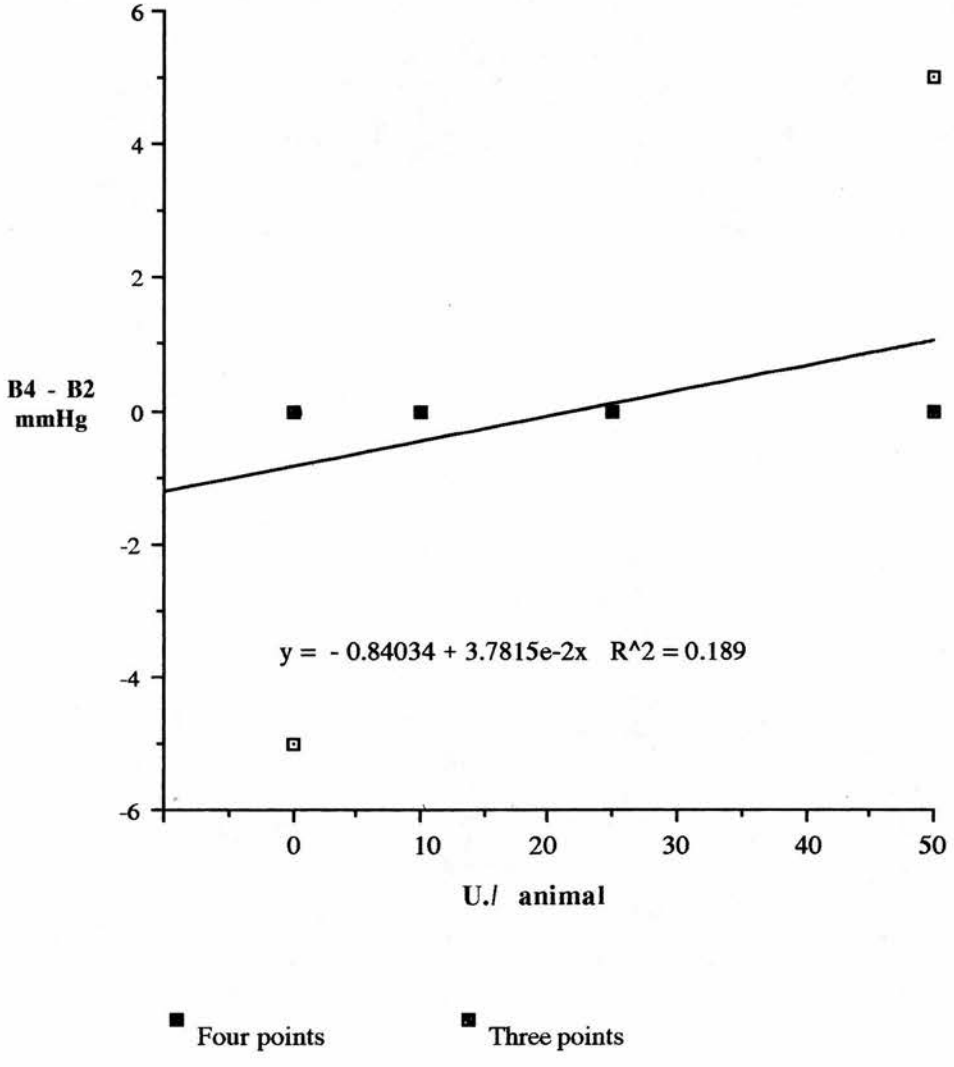
Figure 5.20b. Effect of 0 - 25 U.Calcitonin on Strontium Clearance



Significance of the regression, $p<0.8$.

Correlation coefficient (r) = - 0.06.

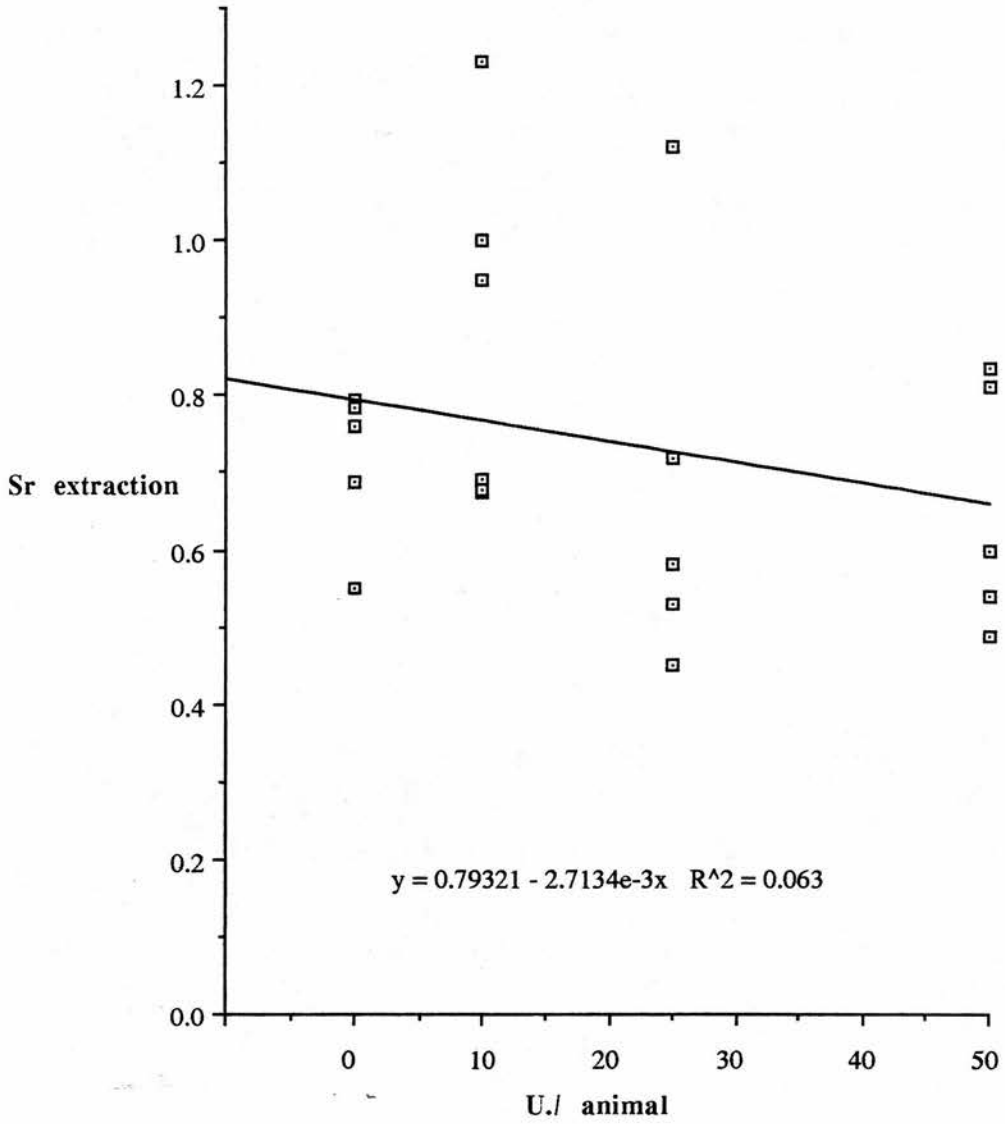
Figure 5.21. Change in blood pressure with Calcitonin treatment



Significance of the regresion, p≤ 0.07

Correlation coefficient (r) = 0.435

Figure 5.22. Effect of Calcitonin on Strontium Extraction



Significance of the regression, $p<0.034$.

Correlation coefficient (r) = 0.46

Figure 5.23. An example of the arterial pressure trace from an animal given 50U. Calcitonin

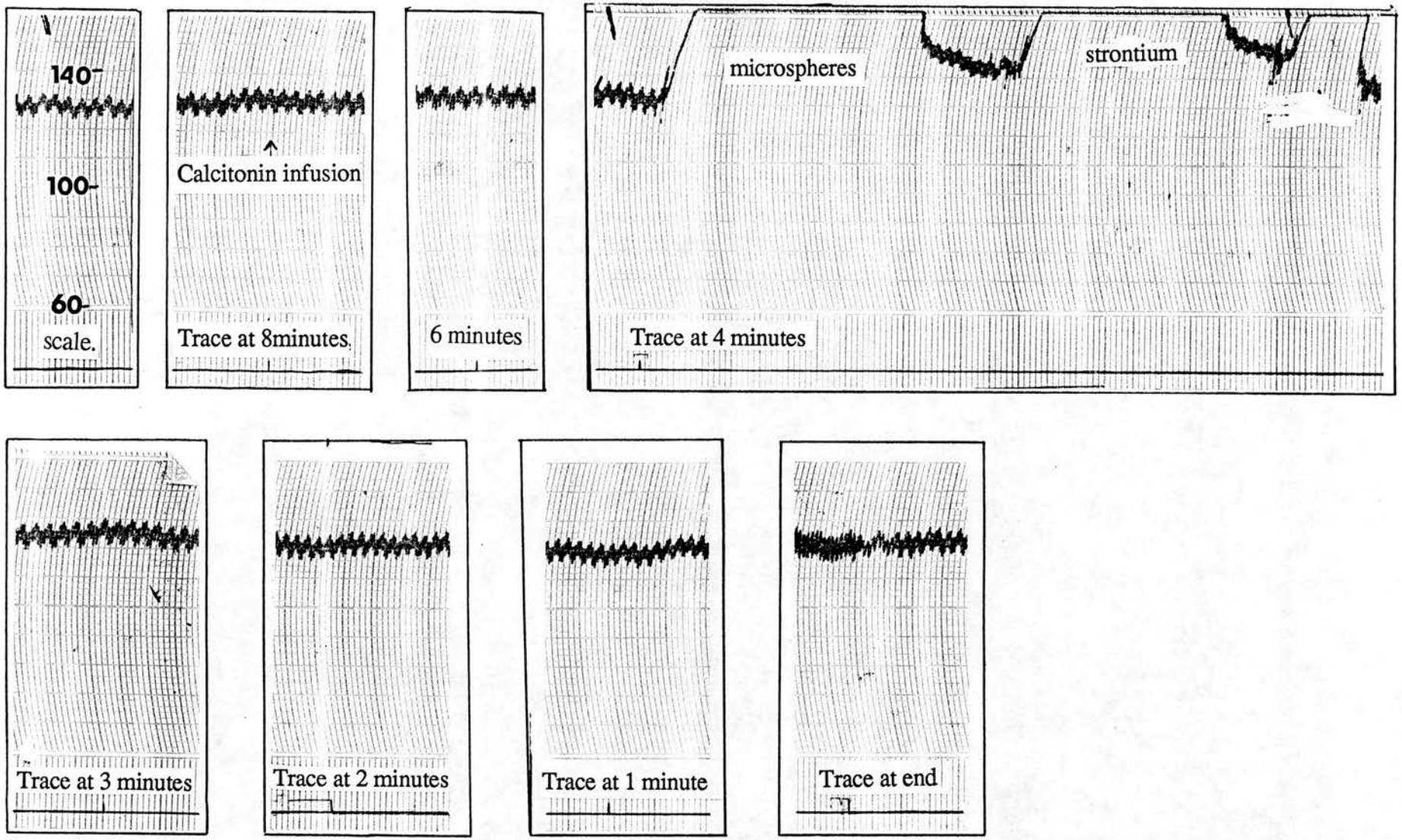
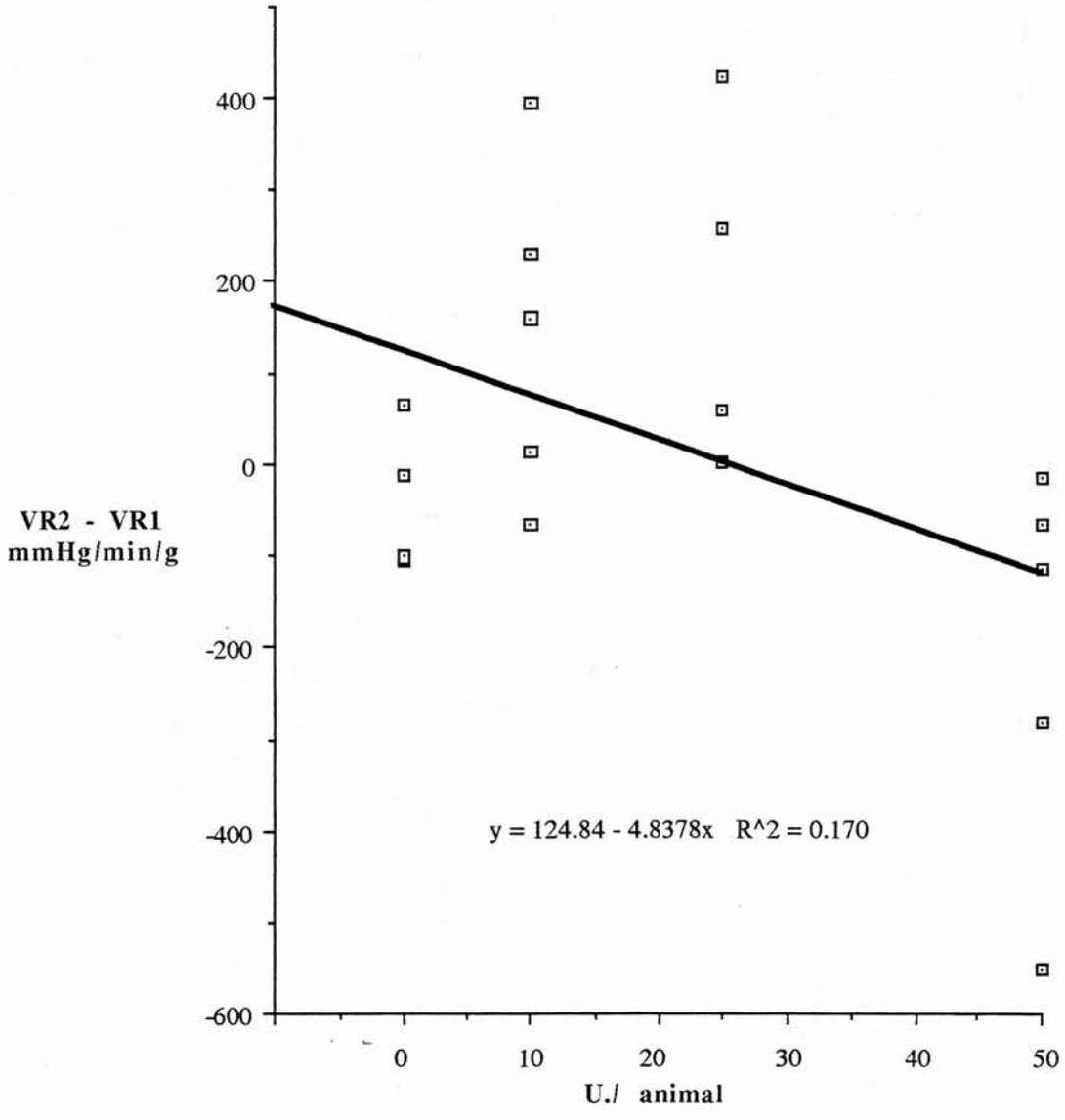


Figure 5.24. **Change in bone vascular resistance in response to Calcitonin**

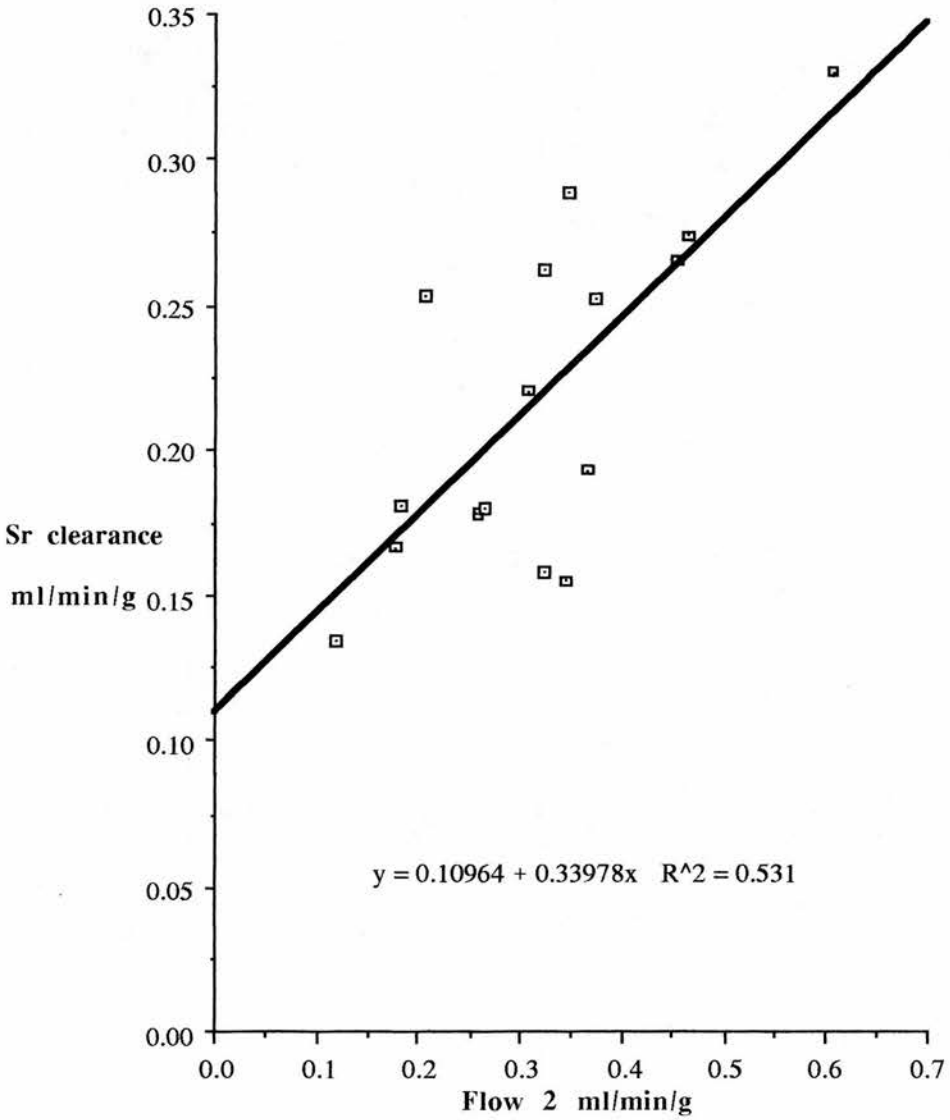


V.R.1 = Vascular Resistance as estimated from cobalt flow and blood pressure (B1.)
V.R.2 = Vascular Resistance as estimated from tin flow and blood pressure (B3).

Significance of the regression, $p \leq 0.08$.

Correlation coefficient (r) = - 0.042

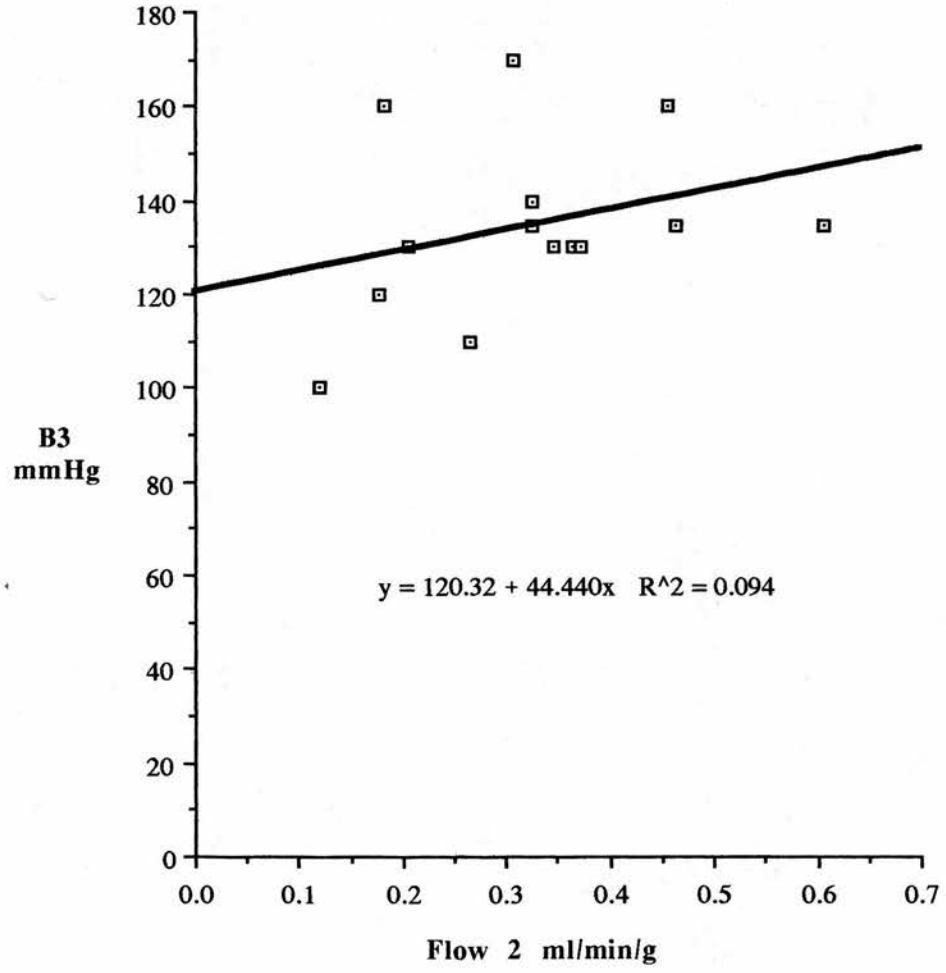
Figure 5.25. Flow versus strontium clearance in the Calcitonin treated animals



Significance of the regression, $p \leq 0.73$

Correlation coefficient (r) = 0.014

Figure 5.26. Flow versus blood pressure in the Calcitonin treated animals



Significance of the regression, $p \leq 0.28$

Correlation coefficient (r) = 0.31

5.6. Discussion

5.6.1. Effect of the calcium regulating hormones

Parathyroid Hormone

In summary PTH treatment resulted in two significant changes.

1. A significant reduction in bone blood flow.

2. A similar decrease in arterial blood . pressure.

Both of these decreases occurred in a dose dependent fashion, that is an increase in concentration caused a decrease in both flow and pressure. Vascular resistance in bone is significantly affected at both the 4 and 8µg doses, a reduction when compared to he control values, however at the 12µg dose the level returns to normal.

Hypotensive action of PTH

Biochemical changes in target tissues in response to PTH can be detected within minutes. Similarly the change in arterial blood . pressure seen during this study occurs soon after administration of the hormone and this reflects a vasodilatatory effect which would cause an increase in systemic blood flow.

The vasodilatatory action of bPTH(1-34) has been demonstrated in the rat by Pang et al (1980a.), the mean arterial blood pressure was shown to decrease in a dose-related fashion in the intact animal. This effect lasted approximately two minutes at the lowest dose and at the higher doses for twenty minutes or longer. This lengthening of recovery period with increased dose was also noticed in the work presented here. Pang compared three preparations all showed a dose dependent effect but the extent varied depending on the product, at the highest dose (about 36 I U. per 350g animal, which is equivalent to 6µg) the average change was approximately 40mmHg. This value falls between the the changes shown at 4 and 8µg doses used in this study, this could be expected as the dose falls between these two values. A later study by Pang et al (1983) investigated further this hypotensive property comparing its timing with that of the hypercalcemic

response. The concentrations of PTH used by Pang et al are all less than the than those used here (i.e. the maximum dose is approximately 1.2µg compared with the lowest dose of 4µg used in this study) but all produced hypotensive effects. The length of this hypotensive response was only minutes and considered to be separate from the hypercalcemic effect.

These papers confirm the findings of this work, bPTH(1-34) has vasodilatatory effects, at least on the general circulation (a reduction in the arterial blood pressure), that is immediate and the duration of the response depends on the concentration administered. This dilatation does not occur in the bone vascular bed, which is indicated by the lack of change in the vascular resistance.

Driessens and Vanhoutte (1981) investigated the response to PTH in a perfused dog tibia , failing to find any significant change in perfusion pressure in response to treatment and no direct effect on the vascular smooth muscle cells of the bone blood vessels was observed. Similarly McCarthy et al (1986) found no effect on perfusion pressure. These authors performed the perfusion at a constant rate and thus perfusion pressure was proportional to motor tone. Parathyroid hormone therefore appears to have no effect on the vascular tone in the canine tibiae even though some bone vessels have a lining of vascular smooth muscle.

The evidence would suggest that PTH has a vasodilatory action on the peripheral circulation but not on the bone vascular bed. Vascular resistance in bone remains constant following PTH treatment indicating that no dilatation or constriction has occurred. The decrease in bone blood flow seen after PTH can be related to the drop in arterial blood pressure.

ie. Flow = arterial pressure ÷ resistance

Thus this effect on flow reflects a reduction in pressure rather than any constriction of the blood vessels.

Hypercalcemic action of PTH

Parathyroid hormone also has an effect on plasma calcium levels, some authors suggesting that this action is distinct from its effect on blood pressure. The timing of this response is very different from that of the hypotensive response, the effect taking days in the rat (Stevenson 1983). However changes in calcium transport have been detected within minutes giving rise to hypercalcemia, using perfusion of isolated bone (Parsons and Robinson 1968).

Strontium-85 has been used as a calcium analogue in this study thus any direct effect of PTH on calcium will be reflected in a change in strontium clearance. The results of this study show that clearance in the PTH treated group is flow dependent, a decrease in flow having an associated decrease in clearance, although this reduction is not significant. Correlation between flow and clearance is also demonstrated by the untreated animals and this would infer that the effect on strontium clearance is a normal response to reduced flow and does not arise independently as a result of PTH treatment.

Shaw and Dacke (1985) have demonstrated that PTH causes an inhibition of calcium movement into bone at short time intervals. They suggest that the primary hypercalcemic response is mediated by a rapid decrease of calcium exit from blood. This can be interpreted in terms of inhibition of calcium entry into bone rather than by more classical osteolytic and renal effects. Although these authors suggest that this inhibition could be reconciled with the plasma calcium pump theory of Talmage they do consider that a vascular mechanism could be involved.

The findings of the present study suggest that alteration in flow does indeed play a part in determining the uptake of the calcium analogue. There is however no evidence supporting the view that PTH acts directly on clearance through an inhibitory mechanism. Boelkins et al (1976) demonstrated that changes in blood flow to leg bone in hens, following PTH injection parallelled changes in plasma calcium and phosphorus concentrations. Further supporting the idea that a change in calcium levels occurs following a decrease in blood flow.

Strontium clearance has been found to vary with flow but the reduction in this is not significant, suggesting that plasma levels are not greatly increased by this effect. The elevation of calcium levels in hypercalcemic states may occur through a second mechanism after the initial vasodilatatory action. However the hypotensive action of PTH results in some change in plasma calcium but either this is insufficient to give rise to hypercalcemic and/or the kidney is able to compensate for the change. This can be reconciled with the work of Pang et al, these authors claim that the two actions of the hormone are distinct and caused by different portions of the hormone and provide conclusive evidence supporting this. The hypercalcemic action increases calcium levels in the plasma through mobilisation of calcium from bone, requiring time to develop while the hypotensive action is immediate with no associated affect on plasma calcium levels (Pang et al 1983). Here the hypotensive action can be seen to occur in the rat with no significant effect on strontium clearance, indicating that plasma levels are likely to be unaffected. Vasodilatation of the peripheral circulation gives rise to a significant decrease in bone blood flow because of a drop in arterial blood pressure even though the bone vasculature is apparantly unaffected. This decrease in flow results in associated reduction in strontium clearance, the two variables correlated to some extent (r=0.83). Clearance is not significantly affected by PTH treatment. This therefore does not support the theory of Shaw and Dacke that an inhibition of calcium entry into bone at short time intervals (beginning at 3 minutes) produces hypercalcemic effects. The lack of effect on strontium levels suggests that no primary hypercalcemic response is demonstrated by any of the PTH treated animals in this study. This is in direct contrast to the decreased net 45-Ca uptake into rat femur only three minutes after injection shown by Dacke and Shaw (1987) using similar concentrations. A decrease in blood flow should alter the clearance of any tracer, the blood flowing through capillaries at a slower rate increasing the transit time and allowing larger quantities of the molecule to diffuse across the wall, assuming that the transport is flow-limited. If however the rate is still to high to be flow-limited than this transport will be diffusion limited. The control

animals in this study show a relationship between flow and clearance that appears to be flow-limited (Figure 3.9.). That is a change in flow is reflected by change in clearance even at the higher flow rates. The PTH animals demonstrate a similar relationship (Figure 5.9.) suggesting that any effect on flow will result in a change in clearance supporting the view that at five minutes any effect on calcium levels arises because of a vascular effect.

The findings presented here suggest that PTH acts on the peripheral vasculature causing vasodilatation and hypotension. This gives rise to a systemic decrease in blood flow that is reflected in the decreased arterial pressure. In bone, blood flow is reduced because of this decrease in arterial pressure while vascular resistance remains unchanged. Clearance appears to be unaffected by PTH treatment and therefore this work does not support the theory of an inhibitory mechanism operating in the uptake of calcium by bone during the hypotensive effect. This mechanism may play a part in the hypercalcemic action but this was not seen at the short time intervals of this work.

Prostaglandin E2

In summary prostaglandin E2 treatment produced a significant effect on:

1. bone blood flow (dose dependent decrease),
2. strontium clearance (similar decrease with increasing dose),
3. arterial blood pressure (reduction),
4. strontium extraction (a dose dependent increase) and
5. vascular resistance (a similar increase).

Hypotensive action

Like PTH it has been demonstrated that PGs have a hypotensive action in most species and in most vascular beds (McGiff et al 1976, Messina et al 1976) its hypercalcemic action is however debated, here the former is discussed with reference to the above findings.

The results of this study agree with the findings of a hypotensive effect. The arterial blood pressure is decreased at all doses. The range of this indicates that the extent of effect is similar over the selected concentrations (47% at 20µg to 43% reduction at the maximum dose). As in the PTH animals the duration of this drop in pressure increases with dose.

This hypotensive effect has been shown to result from the vasodilatatory actions of PGE2 (Messina et al 1976). Therefore all things being equal the vasodilatation of blood vessels should cause an increase in blood flow with a reduction in the systemic vascular resistance. The present results however show the exact opposite, a significant decrease in bone blood flow with increasing dose of the agent. Examination of the values for bone vascular resistance provides an explanation for this. In bone the resistance to flow increases thereby reducing the flow rate. Thus although there is a drop in arterial blood pressure as a result of the vasodilatatory action of PGE2 this does not occur in bone. It has been shown that PGE2 does not cause vasodilation in all cases. In fact constrictor effects have been noted at selected sites (Goodman and Gillman 1980).

Blood flow is measured at only one time period ie. at four minutes as the entrapment of microspheres is almost immediate, this therefore does not reflect the maximum effect on blood pressure. At the point of microsphere injection the reduction in pressure is 37% at the 20 and 40 microgramme doses which increases to 46% at the 60 microgramme level, but it is only 28% at the highest dose. Therefore the vasodilatatory effect on the systemic circulation requires time to reach it's maximum,The duration of this response varies with the dose.

Prostaglandins had been shown to have potent vasodilatatory effects as early as the 1960's (Bergstrom et al 1968, Solomon et al 1968), though the quality of this biological action varies with the individual prostaglandins. The work of Bergstrom et al (1964) demonstrated that single injections of PGE1, PGE2 and PGE3 all caused a significant temporary fall in blood pressure during the continuous injection of norepinephrine. This represented a drop of 30mmHg for 5µg PGE2 and 40mmHg for 10µg PGE2 per animal (approximately 30% and 40% drop in each case). They found that this pressure drop was of a short duration lasting only minutes, unlike its effect on plasma free fatty acids which lasted for longer periods. These results therefore support the findings of the work presented here. The variation in the degree of effect and duration may reflect the choice of animals (i.e. Bergstrom used dogs of 14-25kg) and also the different sources of prostaglandins. When comparing the biological activities of four prostaglandins Horton and Main (1963) discuss the effect of PGE1 and PGE2 on blood pressure and blood flow. In both cases rabbit blood pressure was reduced, a threshold effect occurred at concentrations of 600ng/kg.

It is clear therefore that certain prostaglandins have depressor actions.This study highlights the vasodilatatory action of PGE2 in the systemic circulation of the rat. This results in a significant reduction in arterial blood pressure. The peripheral flow does not however appear to be significantly affected by this action (there is no change in muscle flow). The bone vascular bed does not exhibit this vasodilatatory response, bone vascular resistance remains constant. The effect of PGE2 on bone blood flow therefore reflects the change in the arterial pressure.

Hypercalcemic effect

Both the hypercalcemic action of PGE2 and its ability to stimulate bone resorption in vitro have been well established. This would suggest that this agent should be capable of producing hypercalcemia in vivo.

The results of this study support the theory that PGE2 raises plasma calcium levels, a change in strontium clearance reflecting the drugs' effect on calcium transport. Strontium clearance has been shown to be flow dependent in the control animals, a comparison of the slope of this group (b=0.47) with the PGE2 treated animals (b=0.78) shows that the two lines are statistically different. Confirming that mineral clearance is in some way affected by PGE2, the drug having a direct effect on strontium clearance independent of its action on bone blood flow. These findings can be reconciled with the work of Shaw and Dacke (1985) who have suggested that PGE2 causes primary hypercalcemia, possibly through inhibition of calcium entry into bone. The doses used in this paper are comparable with the concentrations used here therefore the reduced clearance could result from an inhibitiory effect.

Other studies have not found a hypercalcemic reponse to PGE2 treatment. Robertson and Baylin (1977) failed to produce a hypercalcemic response in the intact rat as did Klein and Raisz (1970) in parathyroidectomized animals. In both these papers the doses of PGE that have no effect are less than in the present study but Klein and Raisz did find a small increase at the 40µg dose (200g rats).

The bone resorptive effects of PGE2 in vitro may be influenced by endogenous substances in vivo and therefore do not demonstrate so potent an effect in the intact animals. Or this lack of effect could arise because in vivo the levels of PGE2 are elevated locally and specific to the site of effect. Thus to have the same action in vivo the blood levels may be required to be sustained at high concentrations, increasing local levels of PGE2. Evidence supporting local prostaglandin-stimulated bone resorption in vivo has been documented by Goodson et al (1974). These authors found that repeated injections of PGE1 directly on rat calvarium produced changes in bone morphology linked to increased bone resorption.

The effects of PGE2 in this work is only examined over a short time period i.e. five minutes, and the morphological changes require more time to occur. Therefore it is unlikely that increased bone resorption causes the changes in mineral clearance observed. The doses used in this study could be adequate to produce the local levels in bone necessary for action in vivo. Thus the effect on clearance levels may reflect a hypercalcemic action of the hormone but the calcium levels were not measured in these animals and thus it was not established that hyperalcemia occured.

Prostaglandin E2 has a direct effect on bone, decreasing strontium clearance independent of its effect on bone blood flow. This effect would appear to be flow independent, but whether this is through action on capillary permeability or osteocyte metabolic activity is not clear. These results support the suggested "novel" inhibition of calcium uptake in response to PGE2 but do not confirm any hypercalcemic action of the hormone in vivo.

Calcitonin

Administration of calcitonin resulted in only one significant effect, an increase in strontium clearance. This change is however dependent on the effect shown in the 50U. group, representing a 30% increase when compared with 0 while at the other doses clearance remained constant (i.e. 100% and 95% respectively). Similarly an increase in bone blood flow is shown at the 50U. dose (42% increase) that is not apparent at the other doses (10U.-- 20% reduction, 25U.-- 30% reduction).

Effect on arterial blood pressure

In this study calcitonin has no effect on arterial blood pressure, the recordings remaining relatively constant during the entire test period no matter the dose of drug. This is similar to the findings of Porter et al with regard to perfusion pressure. These however differ from those of Driessens and Vanhoutte (1981), who infused calcitonin into the isolated tibia after cannulation of the nutrient artery. The doses used in the present study are comparable to the higher doses used in Driessens experiments but in an intact animal as opposed to the isolated bone, possibly this gives rise to the different findings. Comparing the papers of Driessens and Porter the former experiments involved denervation of the tibia which perhaps increased sensitivity to the hormone and thus account for the difference in findings.

In the rat calcitonin has no effect on the peripheral circulation indicated by the lack of effect on arterial blood pressure. However bone blood flow is reduced at 10 and 25U. ($p<0.05$ DF=16)) suggesting that bone vasculature is in some way affected, possibly through specific action on bone vascular tone. Therefore although calcitonin does not appear to affect vascular tone in the general circulation this reduced flow and the evidence of Driessens and Vanhoutte supports its action in the bone vascular bed, resulting in a reduction in bone blood flow in the rat. Calcitonin has been shown to cause receptor loss resulting in an inability to respond to further stimulation. This escape phenomenon could be what is occurring at the 50U. dose thereby negating the constrictor

action of the agent seen at the other doses. In vitro this escape occurs between 16-24 hours, this timing is however well out with the duration of the present experiment. The 'escape ' phenomenon may be an important aspect of calcitonin action in vivo but it is unlikely that it is of importance in this study. Therefore the increase in flow and strontium clearance shown in this study must arise through another mechanism.

An examination of the pressure traces shows that the heart rate increases with the 50U. dose this may result from a pharmacological effect and could give rise to a general increase in blood flow which would in turn elevate the bone blood flow. This could therefore explain the increased blood flow seen at this dose i.e. a pharmacological effect causing an increase in the rate of cardiac output elevating the general blood flow rather than any dilatator effect in the bone blood vessels.

Reviewing the results it appears certain doses of calcitonin affects bone blood flow thereby increasing bone vascular resistance and this findings agree with those of Driessens. However as a whole bone blood flow is unaffected by calcitonin because of the increase shown at the 50U. dose. In order to support the pharmacological effect of higher doses further work could be done to incorporate higher doses of Calcitonin into the study.

Plasma calcium levels

Calcitonin has been shown to have hypocalcemic effects, reducing calcium levels in the blood by retaining the mineral in bone rather than promoting its deposition. There is no evidence that calcitonin stimulates uptake of calcium into bone. The lack of significant effect on strontium would support the view that calcitonin does not actively increase calcium uptake. Additionally it is possible that the rat does not remodel bone extensively and in the adult bone turnover would be even more limited thus any hypocalcemic effect would be small and not necessarily appear in these experiments. In the mature animal it appears that calcitonin inhibits bone resorption but the effect on plasma calcium is only seen in situations where bone resorption contributes significantly to the maintenance of normal serum levels (Martin 1983).

At the 50U. strontium clearance is increased this reflects the change in flow also seen at this dose, the increase in flow resulting in an increase in clearance since the calcitonin animals demonstrate the same relationship between flow and clearance as in the control animals. In fact examination of the plot of the control animals suggests that a clearance value of 0.26 would be appropriate for a flow 0.41ml/min/g thus supporting the view that this increase in strontium clearance results from the change in flow. Strontium extraction does however decrease in response to increased dose but this is not significant and at the 10U. dose the mean value is greater than the control mean. Since this is dependent upon both flow and clearance this reflects the change in flow at the 10 and 25U.doses, and the change in both variables at 50U.

Therefore over the dose range calcitonin appears to have a no direct effect on bone, though bone blood flow is increased possibly through constriction specifically in the bone vascular bed at the 10 and 25U. dose. The agent also has no effect on mineral transport. At the 50U. dose the effects of the agent on flow are opposite to those at the other doses, this may arise through an increased cardiac output but because the activity of the microspheres were not estimated before administration this could not be confirmed by this study and is an assumption.

5.6.2. Mechanism of action

All these agents have been shown to affect the production of cAMP and the role of this as a secondary messenger in the cellular response of these agents has been widely investigated. This is of relevance to the transport of minerals but PTH and PGE2 also has hypotensive actions and therefore this section deals with the mechanism of the hypotensive response and that of the cellular response.

Mechanism of hypotensive action

Parathyroid hormone.

This work shows that PTH has a vaso-depressor action in the rat; this vasodilatatory effect is dose dependent and causes a reduction in blood pressure. Therefore the action of this agent on the general circulation and specific capillary beds is important when considering the mechanism of this dilatation.

The vasodilatatory action of PTH is specific to particular target vascular beds, the hormone acting directly on the vasculature. Pang et al (1980b.) failed to show dilatation in the skeletal muscle of dogs although such vascular beds as the heart, kidney, liver and stomach did show this response. A further study in 1982 (Pang et al) puts forward the theory that PTH has a direct vasodilatatory action on vascular smooth muscle. The dilator response in bone to ATP appears to be through action on the vascular smooth muscle (Gross et al 1979). If vascular smooth muscle is involved in the response to PTH then vasodilatation of bone blood vessels could occur through the same mechanism as ATP. Parathyroid hormone causes vasodilatation in the general circulation and in specific vascular beds but in bone this effect is questionable. The systemic pressure decrease and dilatation of the peripheral blood vessels could lead to a change in blood flow in the general circulation.

Therefore the question is does PTH cause vasodilatation of the blood vessels in bone? Parathyroid hormone is a potent vasodilator with specific beds as its target. These vascular beds all have quite high blood flows rates, and the dilation of their vessels would

result in almost an immediate decrease in blood pressure, as seen in this study. The vascular bed of bone does not fall into this category and this would suggest that the vasodilaattory action might not occur. The hypotensive action of this hormone is not significantly diminished in the presence of a and ß adrenergic, H1 and H2 histaminergic receptor antagonists (Pang et al 1980a, 1980b). Thus the action of PTH on the vasculature is direct and not mediated by local vasoactive substance. Within bone the blood vessels have a vascular smooth muscle component until they divide to form marrow sinusoids. If the PTH causes dilatation of vascular smooth muscle in bone one would expect this to alter the response of these cells to constriction caused by noradrenaline, this is not the case (Driessens and Vanhoutte 1979). The evidence of McCarthy et al (1986) agrees with these findings, although these are in apparent disagreement with the results of Pang et al. There are numerous explanations that could account for this. Firstly most of the other work has been on systemic vessels and not bone blood vessels, thus only assumptions can be drawn as to the action of the hormone on bone vessels without supportive evidence in situ. Secondly it has been previously demonstrated that vascular smooth muscle cells have heterogenous responses to vasoactive hormones (Vanhoutte 1978). Thus the response in one vascular bed need not reflect that in another. Alternatively in vivo PTH may exhert a different action than when added directly to the bone circulation.

This however does not answer the question, and unfortunately the evidence is not conclusive as to the effect of PTH on bone vessels in vivo. It is apparent that in the perfused situation the vascular bed of bone shows no response and the lack of effect on induced constriction suggests that there is no vasodilatatory response. Here the vascular resistance of bone remains constant indicating that neither a constrictor or dilator effect occurs in response to PTH. The change in bone blood flow seen in this study reflects the reduction in arterial blood pressure and this is sufficent to reduce the flow significantly even though vascular resistance remains constant.

Prostaglandin E2.

Like PTH this hormone causes a dose dependent reduction in arterial blood pressure. This occurs because of vasodilatation of the peripheral blood vessels, causing an increase in systemic blood flow. This systemic vasodilatation however appears to contradict the fall in bone blood flow. Therefore PGE2, at least at the higher doses, may have a direct constrictory action in the bone vascular bed thereby reducing flow through a change in vascular resistance.

Messina et al (1976) reviewed the evidence supporting action of PGE2 in local circulatory control. They found that the prostaglandins are potent vasoactive substances in a variety of regional circulations and have the ability to alter vascular reactivity to a diverse group of vasoconstrictor stimuli, independent of their dilatatory ability. In particular, PGE2 and PGA1 are effective vasodilatator substances in a number of vascular beds, including skeletal muscle. Additionally these reduce the vascular responsiveness to vasoconstrictor agents. The authors infer that the prostaglandins contribute to the control of vascular reactivity through this mechanism of reduced response.

McGiff et al investigated this prostaglandin mediated control of vascular reactivity, finding that prostaglandins are synthesised in the walls of the major resistance vessels, these authors suggest that prostaglandins directly affect the constrictor state of blood vessels. The capacity of blood vessels to generate these agents in their walls is directly related to regulation of vascular tone and reactivity. This would support the view that in vivo the prostaglandins levels are elevated locally and specific to the target site. Therefore the concentrations used in any study should be adequate to reflect the local levels in situ, otherwise the effect may not mirror the actual response to the compound.

Malik et al (1978) show that prostaglandins, in particular PGE2, reduce the vasoconstrictor effect of sympathetic nerve stimulation in the vasculature of rat spleen and pancreas. This vasodilatatory response is caused by action on the adrenergic neuroeffector units, the effect is not only species dependent but also varies between different

vascular beds in the same species. This author also noted that the constrictor action of noradrenaline is inhibited by PGEs', possibly through the same mechanism since the action of noradrenaline is also through adrenergic receptors in the smooth muscle cells of the vessel wall.

It appears therefore that prostaglandins contribute to the regulation of vascular tone and thus arterial pressure through three mechanisms; a) by acting directly on smooth muscle, b) by modulating the activity of the adrenergic nervous system, and c) by influencing the activity of other vasoactive substances. In this study the PGE2 administered would act on the adrenergic nervous system and on smooth muscle. The regulation of this vascular tone appears to be localised, vascular resistance vessels can generate their own supply. Although there is no direct evidence that bone blood vessels take part in this regulation the results of this study would suggests that they do.

Prostaglandin E2 appears to cause vasodilation in the systemic circulation, indicated by the reduction in the arterial blood pressure but its effect on bone vessels is that of constriction. Bone blood flow decreases in repsonse to PGE2 and this occurs because constriction of the vessels increases the bone vascular resistance. The quantities used here could be sufficient to produce the local levels necessary to cause and maintain constriction and therefore reflects the systemic doses required for generation of adequate local concentrations.

Mechanism of cellular action

Of these agents PGE2 is the only drug that significantly affects mineral transport independent of its effect on bone blood flow. It is not however clear whether this action is through action on capillary permeability or osteocyte metabolic activity.

Therefore this section deals with the possible mechanism that would give rise to a cellular action which decreases strontium uptake.

Both PTH and prostaglandin stimulate adenylase cyclase production and increase the bone cell content of cAMP, and it has been suggested that cAMP acts as a secondary

messenger in the bone resorption process. However calcitonin also has an effect on cAMP production in bone cells giving rise to inhibition of resorption. This discrepancy can be explained by examining the actual cell types upon which these agents act and the levels of cAMP produced. PTH and PGE2 act on the osteoblast and not the osteoclast, and calcitonin acts on the osteoclasts but not the osteoblast. High doses of cAMP produced in response to calcitonin inhibit bone resorption by acting on the bone resorptive activity of the osteoclast. Further support for this action of calcitonin is provided by the work of Chambers et al (1982) who demonstrated that calcitonin inhibits the mobility and bone resorption capacity of the isolated osteoclast. On the other hand low concentrations of dibutyl cAMP promote bone resorption and unlike PTH, high concentrations of it inhibits this resorption. Additionally calcitonin inhibits the effect of PTH when administered at high concentrations (Klein and Raisz 1971). Therefore the difference in action between calcitonin and the bone resorbing agents can be explained in terms of the site of the receptors and the levels of cAMP which is required to produce a response in the osteoclast.

This however does not explain the different effects of PTH and PGE2 therefore the relationship between these two drugs requires further examination. Initially these hormones act upon the osteoblast and this action leads to the generation of some signal which leads to the recruitment, maturation and activation of osteoclasts (Martin 1983). The activation of adenylase cyclase and subsequent protein kinase has been well demonstrated for the osteoblast (Partridge et al 1981, Livesey et al 1982). Whether or not these hormones cause similar effects on the osteoclast is yet to be demonstrated. The work of Livesey suggests that the type of protein kinase isoenzyme stimulated depends on the hormonal effector, PGE2 and PTH stimulate different types depending on the cell source. Therefore it could be possible that the elevation of cAMP levels with in the osteoblast mediates the response of the osteoclast.

The morphological response to PTH and prostaglandin has been widely investigated and a comparison of these findings can identify the possible different effect of PTH and

PGE2 on strontium clearance. The effects of these agents are similar, both compounds increase the size and number of the osteoclasts as well as the area of ruffled borders (Hannsjorg et al 1978). Similarly both agents have an effect on the release of lysomal enzymes from bone. Dietrich and Raisz (1975) studied these morphological effects attempting to quantify the extent of each compounds effect on these changes. The administration of PTH affected the numbers of osteoclasts more so than their individual appearances. In contrast PGE2 produced cytological changes that are associated with increased activity of individual osteoclasts in addition to an increase in cell numbers. This increase in metabolic activity could result in a change in plasma calcium levels reflected by the decrease in strontium clearance. McCarthy et al (1986) suggested that the efflux of certain ions from the exchangeable mineral pool was dependent on the metabolic activity of bone cells. The use of a metabolic inhibitor significantly affected the net extraction of strontium-85 but not the maximum instantaneous extraction. This latter parameter is dependent upon the flux of molecules from blood into bone fluid therefore the inhibition of metabolic activity does not effect this influx but may effect the efflux. This is supported by the change in net extraction, strontium does not pass back to the blood to the same extent as in the controls. If this is the case an increase in this metabolic activity in response to PGE2 should cause an increase in the efflux thus decreasing the clearance of strontium.

The above suggests that although PTH and PGE2 stimulates cAMP production in the osteoblast the signal to, or the signal read by the osteoclasts are different, giving rise to differences in the morphological effects. Prostaglandins and PTH have similar effects on bone resorption and the absence of any synergic or inhibitory interaction, and the fact that no further resorption is obtained when the two compounds are combined indicates that they act through a common cellular pathway to elevate cAMP concentrations (Klein and Raisz 1970). Hayes et al (1980) have suggested that different hormones acting via cAMP may trigger different chains of metabolic events in the same cell. This could arise because of a possible difference in the regulation of intracellular cAMP levels by PTH

and the prostaglandins (Rao et al 1977). Alternatively the receptors for PTH and the prostaglandins could be different (Chase and Obert 1975). Therefore the evidence supports the view that the actions of PTH and PGE2 are dependent on the difference in receptors or in the message transmitted from the osteoblast to the osteoclast. Alternatively there may be two cAMP activated antagonistical systems regulating bone resorption, one of which is regulated by the prostaglandins alone (Klein and Raisz 1970). This theory is however highly speculative.

The activation and increase in osteoclastic activity after PGE2 treatment would give rise to an elevation in plasma calcium levels through increased bone resorption. A similar rise could result from PTH treatment although the timing of this is likely to reflect the difference in effect on the osteoclast cell number rather than the increase in activity which results from PGE2 treatment. The decrease in strontium clearance shown by the PGE2 treated group could reflect this increased osteocytic activity leading to an increase in the efflux of strontium. This however is unlikely, the cytological effects require some time to develop and the plasma calcium levels are not significantly elevated during the time of this study indicating that the efflux of calcium is not altered to any extent. This is supported by the evidence from numerous other studies, the hypercalcemic responses occurring long before the morphological and biochemical changes. Therefore although PGE2 does appear to affect osteocytic activity this would not cause the decrease in strontium clearance seen in this experiment but could reflect the changes in plasma calcium seen at longer time intervals.

Additionally the inhibition of calcium movement into bone can be reconciled with the calcium pump model of Talmage (1969). The theory is that calcium moves passively into bone by diffusion through gaps between the surface osteocytes, a transport system restricted to one side of the cell then pumps the calcium back out of the cell into the extravascular fluid. With reference to the PGE2 treatment there may be a reduction in the diffusion of calcium across the osteocyte through a direct inhibitory mechanism reducing the permeability to the ions. Thus less strontium will be cleared because of this

reduction of ion movement. If the transport mechanism still works efficiently then the extravascular concentration will increase thereby decreasing the concentration gradient across the capillary wall further reducing the transport across the capillary.
Calcitonin also elevates cAMP production but this is in the osteoclast itself and results in the inhibition of bone resorption therefore plasma calcium levels would not be expected to rise and their would be no associated change in strontium clearance.

5.6.3. The relationship between flow and clearance

Comparison of the plots of flow versus strontium clearance for the control and the appropriate drug group results in only one significant difference, that for the control (b=0.47) and PGE2 (b=0.78). This supports the view that PGE2 was having a direct effect on strontium clearance independent of its effect on bone blood flow. Parathyroid hormone and calcitonin have lines of regression that are similar to the control animals and the vasoactive groups. Therefore these two compounds act through the same vascular mechanism as the vasoactive agents, any change in flow reflected by a change in clearance.

5.7. Conclusions

Parathyroid hormone and PGE2 causes vasodilatation in the systemic circulation reducing arterial blood pressure. Vascular resistance in bone remains constant in the PTH treated animals although there is a fall in bone blood flow. This reduction in flow reflects the decrease in blood pressure rather than a constrictor effect of PTH. Prostaglandin E2 significantly affected bone blood flow, resistance and arterial blood pressure. Therefore it appears that in bone, at least, a constrictor effect increases resistance thereby reducing flow. Calcitonin has no real effect on the vasculature as flow is not reduced significantly. Both PTH and calcitonin appear to act through the same

mechanism as the vasoactive agents, a direct or indirect vascular mechanism. The significant effect of PGE2 on strontium clearance could be as a result of some inhibitory mechanism affecting capillary permeability to the ions and so reducing strontium uptake. It is unlikely that any morphological effect would occur in the short time interval of this study which would affect this clearance. But it is obvious that PGE2 has a direct effect on bone, reducing strontium clearance, independent of its effect on bone blood flow.

PLASMA CALCIUM

6.1. Introduction

Examination of the literature with respect to plasma calcium levels and the effect of PTH and PGE2 provides conflicting evidence. There is evidence supporting a hypercalcemic response but whether this occurs after an initial hypocalcemic effect, and the timing of the elevation is cause for some controversy.

Firstly the evidence for the effect of parathyroid hormone. Parsons and Robinson (1971) suggest that entry of calcium into bone occurs very early in response to PTH. Therefore the plasma calcium concentration is initially lowered but this was then followed by an elevation in the plasma calcium levels. Boelkins et al (1975) agree with these findings, the maximum hypocalcemic effect occurring at three minutes followed by a hypercalcemic effect, the maximum effect at thirty minutes. This initial drop in plasma calcium following PTH administration may be due to a membrane effect of PTH, increasing permeability to calcium and allowing an initial flux into the cells (Borle et al 1966).

Other authors agree with elevation of plasma calcium concentration in response to PTH but disagree with the hypocalcemic effect. Grubb et al (1977) found that with PTH, plasma calcium concentration rose for approximately twelve hours in thyroparathyroidectomized rats. They concluded that this increase in plasma calcium is caused by an increase in efflux from the bone compartment which receives calcium from the blood. On the other hand Shaw and Dacke (1985) found a net inhibition of 45-Ca in response to PTH and PGE2 (3-30 min) suggesting that this causes the primary hypercalcemic response. Pang et al (1982,83) attempted to separate the hypotensive and hypercalcemic action of this hormone. These authors found that the hypotensive action was immediate and that the 24-28a.a. portion was essential for this action. The hypercalcemic response required time to develop and that the amino acid portion responsible was that of 1-2.

Thus these studies support the hypercalcemic action of parathyroid hormone suggesting that some time is required before elevation of plasma calcium levels are seen.

The evidence with regards to the hypercalcemic effect of PGE2 is even more contradictory. Vanderweil and Talmage (1979) found that PTH raised plasma calcium levels significantly but that PGE2 showed no increase. Prostaglandin E2 was found to duplicate the action of PTH on osteoclasts but not on plasma calcium control. Similarly the work of Beliel et al (1973) does not show any hypercalcemic effect. This is in direct contrast to the work of Shaw and Dacke (1985) who not only found an elevation in plasma calcium in response to PGE2 but that the effect was longer in duration than with PTH.

6.2. Measurement of plasma calcium levels

The aim here is to investigate whether PTH or PGE2 causes hypercalcemia in the intact animal in the timescale related to the previous experiments. One dose of each was selected as the test concentration, 8μg of PTH was chosen because it significantly affected strontium in the earlier experiments and 60μg PGE2 was chosen because it significantly affected clearance although not extraction. The levels were examined to study whether any effects of these agents on mineral exchange i.e. strontium clearance in bone could give rise to hypercalcemia . Additionally the intention was to compare the timing of the hypotensive effect of these hormones with the hypercalcemic action. Because of restrictions in my home office licence the duration of the experiment was limited to the period that the animal was unconscious without causing it undue pain, therefore if the animal gave any indication of being uncomfortable the experiment was ceased. Thus in some animals the experimental period was longer and there are more blood samples. The animals were prepared as set out in 4.4. the only difference is that the tail cannula is not connected to a withdrawal pump as samples were withdrawn into a 2ml syringe by hand.

A reference blood sample 1ml was withdrawn two minutes before injection of the test solution. Further 0.5ml samples were taken at a range of intervals, details of these times are shown in table 6.1. These samples were centrifuged at 3600rpm for five minutes and the supernatant gently removed with another syringe, making sure none of the red blood cells contaminated this solution. These samples were then used to determine the plasma calcium levels using absorption spectrometry.

Quantitative, colorimetric determination of calcium in serum (Sigma -- Calcium binding reagent and Calcium buffer reagent)

1. Calcium binding agent, o-Cresolphthalein complexone, 0.006%, 8-hydroxyquinoline, dimethylsulfoxide (DMSO) and surfactant.
2. Calcium buffer reagent, 2-amino-2-methyl-1-propanol. 450mmol/L.

The calcium assay solution was prepared by mixing equal volumes of the above reagents. Into two cuvets, labelled test and standard, 4ml of this solution was added. The absorbance of these were then read at 575nm with water as a reference (Unicam SP 600). This is the initial [A] reading for each. Added to the test cuvet and mixed was 0.05ml of the serum sample and 0.05ml of the calcium standard solution was added to the standard cuvet. These samples were then read, with water once again as the reference at 575nm. This is final [A]. To ensure correct absorbance readings these have to be completed within 10 minutes.

Calculation of calcium concentration

$$\text{Calcium (mg/dl)} = \frac{\text{Final [A] test - Initial [A] test}}{\text{Final [A] standard - Initial [A] standard}} \times 10$$

6.3. Results

The effect of both PGE2 and PTH treatment on plasma calcium levels are detailed in Table 6.1., which also includes the control data.

(a) Control

This group consists of two animals, from which four separate samples were obtained. Unfortunately one blood sample was lost during the centrifuge process thus the mean and standard deviations are derived from seven readings. All but one value is within the range for normal human levels i.e. 8.5 to 10.4mg/dL. Therefore it seems acceptable to set this as the range for the rat, any values above this would result from raised calcium levels.

(b) Parathyroid hormone

This group consists of four animals, in two the level at five minutes are above that set as normal plasma concentration. This may have arisen through contamination of the samples with red blood cells and examination of the supernatant confirmed the likelihood that this had occurred. The solution was discoloured to some extent, possibly through leakage of red blood cells during the centrifuging process, resulting in high absorption readings. The subsequent values support the view that these are not true readings, the lack of elevation in concentration at these later time intervals suggesting that the blood calcium remains relatively constant. In the other two animals the calcium levels are elevated at forty minutes (in one case this elevation occurs at 20 minutes) these values are even higher than the readings for the contaminated samples. This would suggest that these values reflect an elevation of plasma calcium concentration rather than incorrect readings.

These results suggest that plasma calcium concentrations affected by this dose of PTH, at least at forty minutes. Thus during the five minute period of the bone blood flow experiments it is unlikely that the plasma calcium levels are elevated and this is supported by the lack of effect on strontium clearance.

Table 6.1.Effect of PTH and PGE2 on plasma calcium levels in the rat

Animal No.		1	2	3	4
Control		9.53	8.92	}	
		/	8.91	}	Range 7.0 to 10.05
		10.05	6.96	}	
		8.96	9.92	}	
PTH treated animals					
-2 minutes		9.2	9.1	8.9	9.5
5	"	**10.8**	**10.7**	8.8	8.7
10	"	/	8.5	10.3	9.0
15	"	8.3	/	/	/
20	"	8.3	9.6	**13.1**	9.2
40	"	/	10.0	**14.2**	**11.0**
60	"	/	8.9	/	/
PGE2 treated animals					
-2 minutes		9.9	9.5	9.1	9.1
2	"	9.8	**11.3**	10.2	10.2
7	"	**11.8**	**13.0**	9.7	5.8
15	"	**12.0**	9.9	**11.3**	8.3
60	"	**12.0**	9.4	9.4	9.0

Plasma calcium measured in mg/dl

Assuming a normal range of 8.5 - 10.4mg/dl (acceptable human values) the values in bold are above this level indicating elevated calcium levels.

(c) Prostaglandin E2

This group also included four animals with samples taken up to sixty minutes. In retrospect it was unfortunate that no sample was taken between fifteen and sixty minutes as interpretation of the results is made difficult because of lack of information.

Three of the animals show elevated calcium levels at two minutes if the values are compared to the range of the control animals. The 10.2mg/dL. values are however lower than the normal human levels and in these animals the readings at seven minutes have returned to acceptable normal levels. In three of the animals the plasma calcium levels are raised above the normal human levels (Table 6.1.), suggesting that hypercalcemia does occur. The timing differs between the animals. Therefore it cannot be conclusively said that the plasma calcium levels are elevated within the five minute period of the blood flow experiments. It does however appear that this dose of PGE2 raises plasma calcium levels in the rat and this supports the decrease in strontium clearance found in the other animals. This need not necessarily be sufficient to cause a hypercalcemic effect.

6.4. Discussion

Parathyroid hormone

The examination of plasma calcium levels in four animals shows that the plasma calcium concentration is not elevated within the five minute period of the bone blood flow experiments. This therefore supports the findings of the previous chapter, that is mineral clearance is not significantly affected by PTH treatment.

This work agrees with the findings of Pang et al (1983) as to the distinct separation of the hypotensive and hypercalcemic action of the hormone. At the time period of the bone blood flow work (5 minutes) there is a hypotensive but no hypercalcemic effect. Reduced uptake of calcium suggested by the slight decline in strontium clearance (Figure 5.3.) need not give rise to hypercalcemia immediately. The vasodilatatory effect

occurs immediately. Thus supporting the theory that the two acts are distinct and occur through two separate mechanisms. At the time when the hypercalcemic response is demonstrated (20 - 40 minutes) the arterial blood pressure had recovered indicating that the vasodilatatory action is no longer effective (Figure 6.1.).

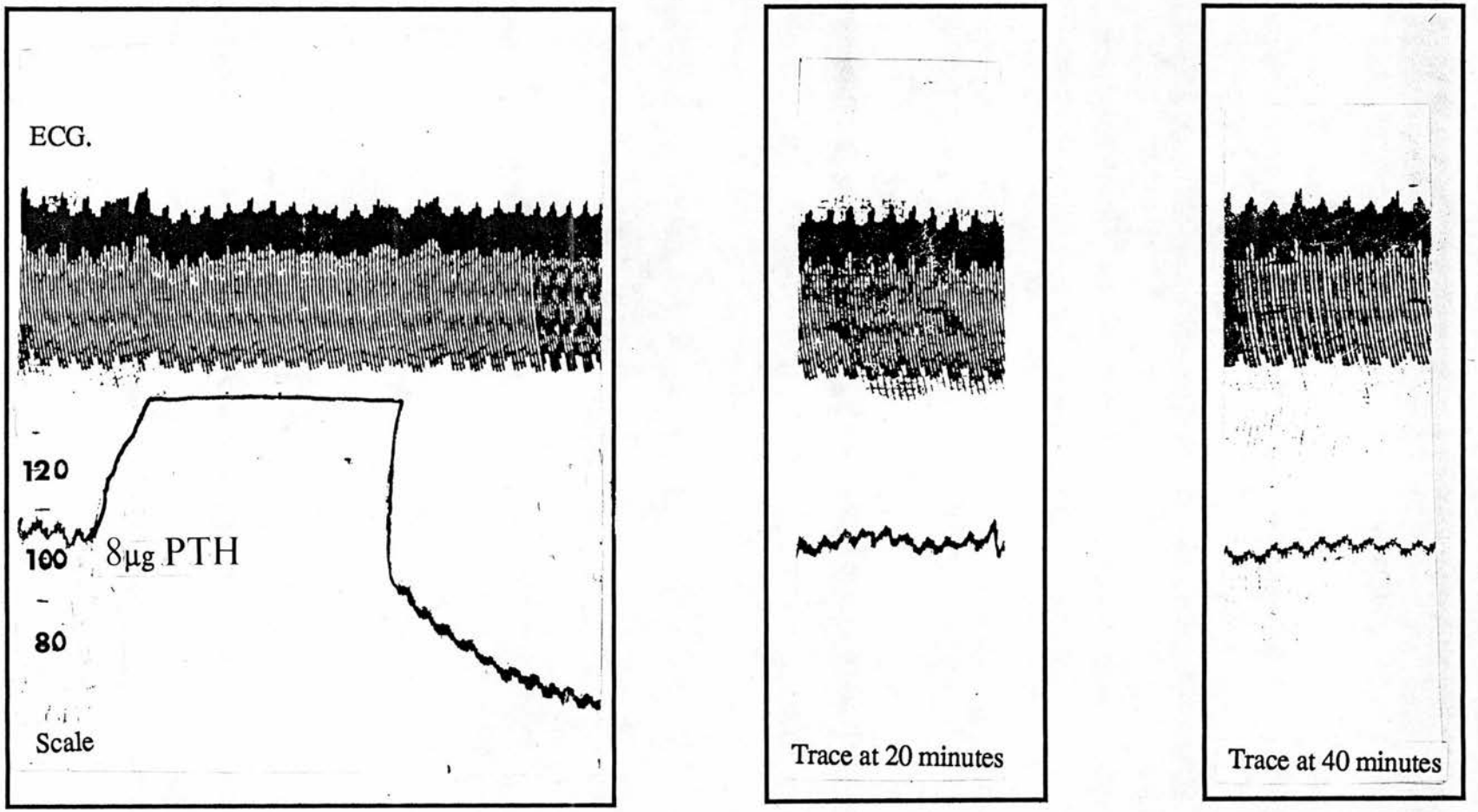
Prostaglandin E2

In general the 60µg dose gives rise to elevated plasma calcium levels but the timing of this hypercalcemic response is questionable.

It is possible that the reduction in strontium clearance shown in the previous section in response to PGE2 treatment (Figure5.13) could reflect this effect on calcium transport. However the data from the plasma calcium study suggests that plasma concentration would be normal during the five minutes of the bone blood flow study. The effect on transport is thus not sufficient to give rise to a hypercalcemic effect within five minutes.

In conclusion this work suggests that in neither the PTH or PGE2 bone blood flow and mineral clearance studies does hypercalcemia occur. Therefore the decrease in strontium clearance seen in the PGE2 study does not reflect a significant elevation of plasma calcium concentration. It may be that this change in calcium levels is so small that no hypercalcemic response is found and/or the kidney is able to compensate for the change and so maintain a relatively constant calcium level.

Figure 6.1. An example of the pressure trace from an animal given 8μg PTH



SUMMARY

7.1. General conclusions

This chapter sums up the findings of the previous chapters, drawing together the relevant facts from each with respect to the objectives set out in the introduction.
Reviewing the introduction the main objectives were ;
1) a review the general vascular supply and architecture of rat bone with particular reference to the blood supply,
2) to establish a protocol to measure bone blood flow and strontium clearance in the rat thereby identifying the relationship between drug concentration and response. In addition to see if any change in blood flow could alter the uptake of minerals or if the exchange of minerals could be altered independently of flow.

The barium sulphate and histological section details the structure of rat bone and supports the general nature of the vascular supply (Chapter 2). That is rat bone consists of cortical and compact bone, with the lamellar bone and Haversian bone present in approximately equal quantities. The x-rays show details similar to that seen in the work of Brookes (1971) i.e. the principal nutrient artery and its ascending and descending branches. The presence of medial canals in the tibia running across the sections is of importance as this indicates that the matrix and cells have a good blood supply following secondary osteogenesis than would normally be expected if only the Haversian canals were present.

After establishing that the blood supply to rat bone allows the use of the microsphere technique to measure blood flow the next section validates the use of this (Chapter 3). This chapter basically sums up the problems associated with the method, most of which arise because of anatomical reasons, i.e. the size of the caudal artery requires a narrow

cannula which can lead to blocking of the cannula or too high a withdrawal rate, resulting in clamping of the caudal vessel. For future studies the important considerations are;

1. that the microspheres have been shown to trap efficiently,
2. the spheres have no haemodynamic effect,
3. the importance of random choice of animal choice and drug dosage in reducing the experimental variation.

Additionally this chapter identifies the possible need of a correction factor in the calculation of flow if the caudal artery is used as a reference sampling point. This work points to a difference in the haematocrit of bone and caudal artery, the actual extent of this difference is questionable, it varies depending on the actual haematocrit of the tail artery in the animal concerned. The suggested cause of this difference is that bone is richer in plasma and poorer in corpuscles than the caudal blood supply. This would lead to streaming of the microspheres, an underestimation of flow and over estimation of strontium clearance. Thus the values for strontium clearance of greater than one in the first set of control animals can be explained in this context. The work is not conclusive and therefore it is impossible to encorporate a correction factor into the actual drug study, only to speculate that one may be required.

The question is therefore: how does the difference in caudal and bone haematocrit identified by this work affects the main study? All the animals are affected by the assumption that the two haematocrits are the same and although this may change the actual results this would not affect the general trends. Thus a 30% correction factor would increase flow in all animals by the same amount and similarly strontium clearance would be reduced in each animal by the same amount. This would mean that the general trends and percentage values calculated for flow and clearance would remain the same. Since the same assumption has been incorporated into each drug group comparisons can be made between each group. The only variable that would be misinterpreted is strontium extraction and since this document deals mainly with the general trends shown by the flow and clearance this is not too important.

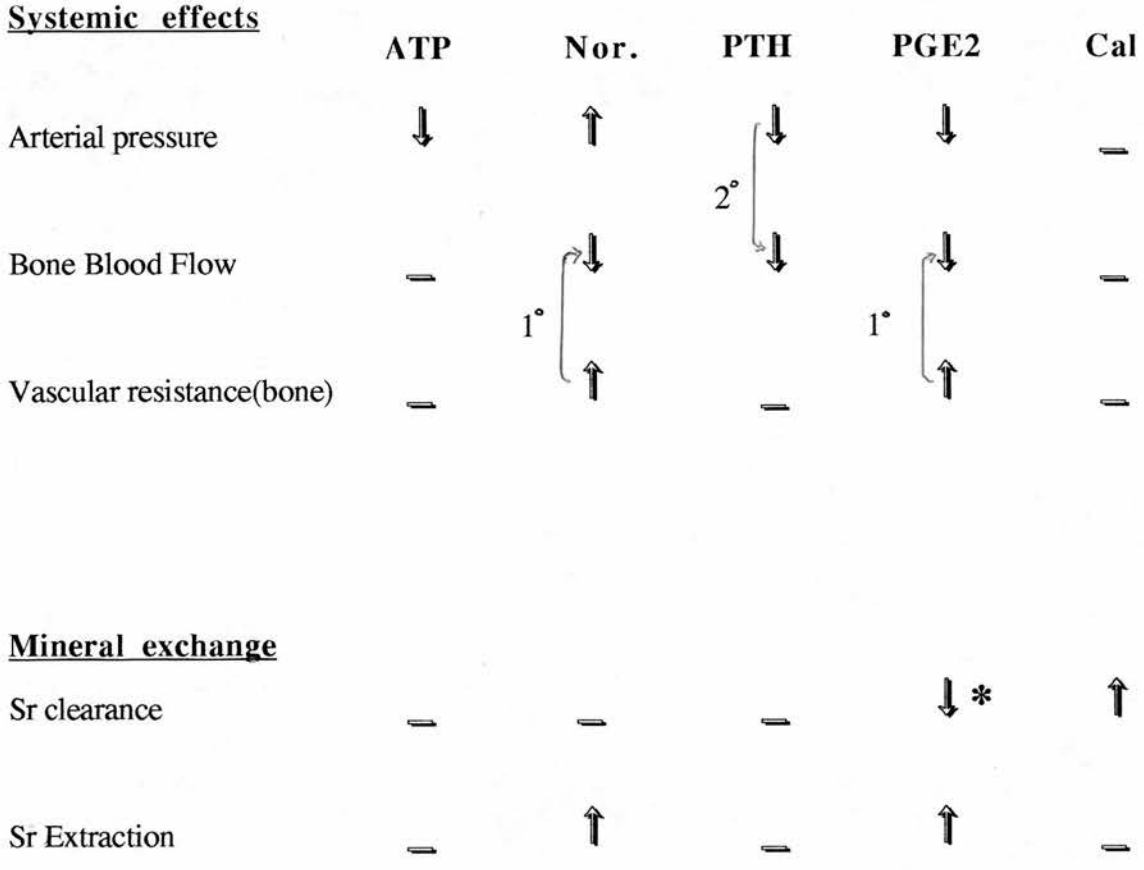
Having established the protocol the actual drug experiments were carried out (Chapters 4 and 5). In each case a the line of regression flow, strontium clearance and vascular resistance in bone along with blood perfusion pressure have been presented. The various findings are summed up in figure 7.1.

With respect to these, the results show that there is a relationship between bone blood flow and strontium clearance in the normal rat (Figure 3.9.). That is clearance varies with flow. The same pattern is shown by the vasoactive agents, ATP and noradrenaline, parathyroid hormone and calcitonin. This similarity in relationship between the animals treated with these compounds suggests that these act through a similar mechanism. This appears to be through a vascular method either directly or indirectly, any change in flow reflected by a change in clearance. Indeed the evidence supports the view that ATP and noradrenaline act directly on the vasculature through alpha adrenoreceptors. Parathyroid hormone also appears to act directly on the vasculature, at least on the smooth muscle component of the systemic circulation. Calcitonin does not appear to significantly affect blood flow or arterial pressure suggesting that it has little effect on the vasculature. Therefore it is likely that it would demonstrate the same relationship to the control animals. The increase in flow seen at the 50U. could arise through an increase in cardiac output and the effect on strontium clearance is similar to that which would be expected in the untreated animal, supporting the view that this change in clearance is a direct effect of a change in flow.

Prostaglandin E2 has a direct effect on bone, decreasing strontium clearance independent of its effect on blood flow. The possibilty of an inhibitor mechanism, preventing the uptake of strontium by the bone seems to be the best option causing this effect. The cytological effect could add to the hypercalcemic effect but this would require hours before any main affected would be seen. These results therefore support the suggestion of Dacke and Shaw (1985) that PGE2 causes a novel inhibition of calcium uptake.

This effect therefore questions the use of bone seeking radionuclides such as 85-Sr, 18-F and Tc-99m in estimating blood flow. Previous work in our laboratory, and by other

Figure 7.1. Summary of the results of the bone blood flow and strontium clearance work



↱ indicates the direction of the vascular response .

The number indicates the degree of the vascular response i.e. 1° = Primary

2° = Secondary

* Strontium clearance is reduced independently of flow.

authors shows that extraction of bone seeking tracers is flow dependent. The present study suggests that in addition the metabolic activity of the bone itself can affect this extraction. This casts doubts on the use of clearance measurements as a method of estimating bone blood flow.

Chapter six examines the hypercalcemic response to both PTH and PGE2. From this work its is unlikely that a hypercalcemic response occurs within the five minute period of the bone blood flow study. Therefore the reduction in clearance seen with PGE2 does not reflect an elevated calcium concentration and does not infer that hypercalcemia has occured. It is possible that the effect is too small to produce a noticible effect or that the kidney is able to compensate for these initail changes in calcium and thus negates the the effect of an increase.

7.2. Improvements to the experimental protocols

Measurement of bone blood flow and strontium clearance

1. In all the animals only assumptions can be made about the increase in cardiac output. With the benefit of hindsight measurement of the activity of the microspheres before and after injection into the animals would have allowed calculation of the dispensed activity and the cardiac output. This would not only have clarified these assumptions but ensured that an adequate dose of microspheres were given to each animal.

2. With respect to the calcitonin animals the increase in cardiac output is suggested by the increase seen in the heart rate at the 50U. dose. There is no indication of this at the 25U. dose therefore in any future studies the use of intermediate doses could be of importance in establishing the dose at which this cardiovascular effect begins. Additionally a larger dose could have been incorporated to study to see if this effect at 50U. is a true response i.e. if the increase in strontium clearance and flow is repeated at the higher doses.

Bone/Caudal haematocrit

This section identifies the possible need for a correction factor to compensate for a difference in the haematocrits at the two sites. Before this can be established certain adjustments need to be made to the procedure set out in this document.

1. The number of animals needs to be increased substantially to minimise the variation that occurs between the animals.

2. Here the arterial haematocrit of the tail samples ranges from 40 to 59% with a mean of 49%. This is in disagreement with the 45% value of Brookes (1965). Therefore this study would have to measure the caudal haematocrit in each animal and use this to calculate the bone haematocrit.

Plasma calcium levels

Here the study can be improved by increasing both the number of animals and the number of blood samples. If possible an extension in duration of the study i.e. over days rather than hours with possible infusion of the hormone or more than one injection of the drug would allow better identification of the timing of the hypercalcemic response. If this was undertaken then the morphological changes could also be examined allowing a comparison of the cellular components with that of normal rat bone.

7.3 In summary this work presents seven main findings

1. The haematocrit of bone and caudal artery are different but this requires further investigation before a correction factor can be calculated .

2. Confirmation that a relationship exists between bone blood flow and strontium clearance in the rat.

3. The results show that a change in bone blood flow can alter the uptake of minerals. Additionally the uptake of minerals can be directly affected independently of blood flow.

4. Prostaglandin E2 appears to cause a dose dependent decrease in strontium clearance independent of its action on blood flow.

5. The PGE2 action could involve a 'novel' inhibition of calcium uptake, possibly through an effect on capillary permeability.

6. The results add weight to the view that clearance measurements are not appropriate for the measurement of bone blood flow.

7. The five minute period of the blood flow study is inadequate to allow a hypercalcemic effect to occur.

7.4. Future work

These experiments highlight the effect of the vasoactive and calcium regulating agents on bone, for example blood flow, arterial blood pressure, vascular resistance etc, but do not attempt to consider the mechanisms by which these agents work. In particular this study shows that the administration of PTH and PGE2 affects mineral exchange. These short term effects have however not been observed in any in vivo experiments. Further studies could therefore be adapted from this work to investigate these effects and the method by which the drugs cross the bone blood barrier.

The concept of a 'bone membrane' has been discussed for some years. Two mechanisms having been proposed to support the hypothesis of the blood-bone disequilibrium. Firstly Talmage (1969) suggests that calcium ions are allowed to diffuse down the concentration gradient (from blood into bone) but these ions are actively pumped out of bone. Secondly it has been suggested that there is a local concentration gradient of solubiliser produced by the osteoblasts lining the resting bone surfaces. The reduction in clearance in response to the PGE2 is independent of blood flow and this can be reconciled with the calcium pump theory of Talmage.

Possible future work

The use of metabolic and calcium blocking agents could be used to attempt to identify the mechanism of these agents action. If active transport is involved in the uptake of strontium from blood to bone in the response to these preparations then the use of a metabolic inhibitor should affect this. Further experiments in whole animal preparations would be unsuitable for this type of study. The use of cAMP inhibitors or other inhibitors could have adverse systemic effects thereby complicating the interpretation of the results even ''killing'' the animal. In vitro experiments could be used as an alternative since the metabolic activity would be controlled and there would be no adverse systemic effects. The removal of the tissue however would likely damage or interfere with any ''membrane'' effect. Tibial perfusion would provide the optimum situation there would be little damage if any to the bone membrane yet the bone could be experimented on in isolation with the drugs having no general systemic effect. The rat however would be unsuitable for this type of study thus another animal would have to be considered. The rabbit is an alternative, the tibial artery can be cannulated successfully.

Suggested experimental protocol for tibial perfusion technique (rabbit/sheep)

1. This technique requires cannulation of the tibial nutrient artery followed by perfusion of the tibial cortex with Krebs Ringer buffer.
2. The ipsilateral femoral vein is then cannulated to provide a site for sampling the venous blood.
3. A bolus of three reference tracers are added via the nutrient artery. These include
    a). 125 I-albumin a reference vascular tracer,
    b). 14 C-sucrose an inert fluid tracer,
    c). 85 Strontium a bone seeking tracer.

    Simultaneously, blood is sampled at the ipsilateral vein at 10 second intervals for 5 minutes.
4. One ml aliquots of these blood samples are then measured for activity.

It is therefore possible to perform two measurements on each animal, the first using standard buffer as a control measurement and the second after injection of the appropriate agent and a calcium blocking agent or metabolic inhibitor. Therefore the results of the animals treated with a metabolic inhibitor or calcium blocking agent and drug can be compared with those of the drug treated groups only. Interpretation of these may highlight a possible mechanism of action.

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APPENDIXES

Noradrenaline Appendix

Mon, Feb 12, 1990 11:48

	µg	F 1	F 2	F2-F1	Sr clearance	Sr Extractiom	B1	B3	B4 - B2	V.R. 1. (Co)
1	0.000	0.358	0.428	7.00e-2	0.299	0.698	85	85	-5	240
2	0.000	0.321	0.332	1.10e-2	0.178	0.536	140	145	-5	436
3	0.000	0.182	0.209	2.70e-2	0.148	0.710	130	80	0	796
4	0.000	0.500	0.477	-2.30e-2	0.336	0.704	115	115	0	230
5	1.000	0.093	0.191	9.80e-2	0.193	1.000	130	145	50	1400
6	1.000	0.524	0.236	-2.88e-1	0.168	0.710	135	190	70	260
7	1.000	0.477	0.315	-1.62e-1	0.183	0.580	160	160	30	330
8	1.000	0.315	0.338	2.30e-2	0.246	0.730	90	90	10	290
9	1.500	0.375	0.183	-1.93e-1	0.172	0.940	190	225	40	505
10	1.500	0.227	0.132	-9.50e-2	0.107	0.810	160	180	30	705
11	1.500	0.479	0.220	-2.60e-1	0.201	0.910	120	140	35	250
12	1.500	0.283	0.223	-6.00e-2	0.210	0.940	150	195	50	530
13	2.000	0.332	0.220	-1.17e-1	0.265	1.200	60	105	45	180
14	2.000	0.364	0.285	-7.91e-2	0.258	0.900	130	170	50	360
15	2.000	0.310	0.145	-1.65e-1	0.138	0.950	100	130	40	325
16	2.000	0.281	0.134	-1.47e-1	0.090	0.700	170	170	20	605

APPENDIX 1

	V.R. 2 (Tin)	V.R.2 -V.R.1	Muscle F1	Muscle F2	MfF2 - MF1	Muscle V.R.1	Muscle V.R.2	Muscle V.R.2 -
1	200	-4.00e+1	0.138	0.084	-5.40e-2	615	1010	3.95e+2
2	480	4.40e+1	0.088	0.084	-4.00e-3	1650	1725	7.50e+1
3	640	-1.56e+2				1930	920	-1.01e+3
4	240	1.00e+1	0.125	0.101	-2.40e-2	920	1140	2.20e+2
5	760	-6.40e+2	0.180	0.110	-7.00e-2	710	1320	6.10e+2
6	805	5.45e+2	0.098	0.181	8.30e-2	1380	1050	-3.30e+2
7	510	1.80e+2	0.136	0.086	-5.00e-2	1180	1860	6.80e+2
8	265	-2.50e+1	0.078	0.073	-5.00e-3	1150	1230	8.00e+1
9	1225	7.20e+2	0.067	0.105	3.84e-2	2835	2135	-7.00e+2
10	1365	6.60e+2	0.075	0.251	1.76e-1	2130	720	-1.41e+3
11	636	3.86e+2	0.016	0.018	1.30e-3	740	8000	7.26e+3
12	874	3.44e+2	0.102	0.108	6.00e-3	1470	1805	3.35e+2
13	476	2.96e+2	0.063	0.121	5.80e-2	950	870	-8.00e+1
14	595	2.35e+2	0.061	0.110	4.85e-2	2130	1550	-5.80e+2
15	896	5.71e+2	0.093	0.144	5.10e-2	1075	900	-1.75e+2
16	1269	6.64e+2	0.100	0.227	1.27e-1	1700	750	-9.50e+2

ATP Appendix

Mon, Feb 12, 1990 11:37

	ATP	F1	F2	F2 -F1	Sr clearance	Sr Extraction	B1	B2	B3	B4
1	0.00	0.297	0.291	0.00e+0	0.197	0.678	160	170	170	170
2	0.00	0.327	0.299	-2.80e-2	0.216	0.723	190	190	190	190
3	0.00	0.136	0.167	3.10e-2	0.122	0.735	180	190	190	190
4	0.00	0.177	0.123	-5.40e-2	0.116	0.943	65	70	70	70
5	0.25	0.235	0.307	7.20e-2	0.179	0.580	100	100	110	90
6	0.25	0.182	0.149	-3.30e-2	0.136	0.913	130	130	100	40
7	0.25	0.258	0.334	7.60e-2	0.301	0.900	95	95	95	80
8	0.25	0.370	0.172	-1.98e-1	0.163	0.950	140	130	130	105
9	0.25	0.286	0.259	-2.70e-2	0.286	1.100	150	130	100	70
10	0.75	0.110	0.091	-1.90e-2	0.137	1.500	120	110	133	90
11	0.75	0.430	0.200	-2.30e-1	0.122	0.610	190	180	135	105
12	0.75	0.469	0.208	-2.61e-1	0.116	0.550	140	120	120	95
13	0.75	0.388	0.137	-2.51e-1	0.087	0.635	150	150	140	105
14	0.75	0.433	0.232	-2.01e-1	0.136	0.584	120	125	90	85
15	1.25	0.174	0.281	1.07e-1	0.182	0.650	150	150	95	85
16	1.25	0.485	0.362	-1.23e-1	0.270	0.740	125	140	100	70
17	1.25	0.587	0.354	-2.33e-1	0.180	0.510	150	130	110	100
18	1.25	0.273	0.203	-7.00e-2	0.170	0.830	130	130	90	80
19	1.25	0.503	0.254	-2.49e-1	0.170	0.570	160	150	105	90

	B4—B2	V.R. 1 (Co)	VR 2 (Sn)	VR 2- VR1	Muscle F1	Muscle F2	MF2 -MF1	V.R.1 muscle	V.R.2 muscle	MV.R.2 - V.R.1
1	0	540	585	45	0.113	0.081	-3.20e-2	1415	2100	6.85e+2
2	0	580	635	55	0.017	0.020	2.10e-3	10920	9743	-1.18e+3
3	0	1100	960	-140	0.119	0.072	-4.70e-2	1260	2220	9.60e+2
4	0	370	560	190	0.311	0.025	-2.86e-1	210	2780	2.57e+3
5	-10	715	670	-45	0.094	0.075	-1.90e-2	1380	1330	-5.00e+1
6	-90	370	240	-130	0.100	0.126	2.55e-2	945	635	-3.10e+2
7	-15	380	750	370	0.123	0.223	9.97e-2	1135	580	-5.55e+2
8	-25	525	390	-135	0.106	0.147	4.10e-2	1415	680	-7.35e+2
9	-60	425	360	-65	0.015	0.079	6.40e-2	6660	1390	-5.27e+3
10	-20	300	580	280	0.098	0.043	-5.43e-2	1950	3140	1.19e+3
11	-75	440	675	235	0.056	0.075	1.90e-2	2500	1600	-9.00e+2
12	-25	290	390	100	0.166	0.066	-1.00e-1	900	1590	6.90e+2
13	-45	380	770	390	0.133	0.423	2.90e-1	940	210	-7.30e+2
14	-40	910	1330	420	0.073	0.090	1.70e-2	1370	1330	-4.00e+1
15	-65	260	320	60	0.137	0.135	-1.50e-3	910	740	-1.70e+2
16	-70	470	440	-30	0.132	0.116	-1.60e-2	1130	950	-1.80e+2
17	-30	260	310	50	0.195	0.169	-2.60e-2	670	530	-1.40e+2
18	-50	320	360	40	0.097	0.206	1.09e-1	820	510	-3.10e+2
19	-60	860	340	-520	0.127	0.166	3.90e-2	1180	570	-6.10e+2

PTH Appendix

Mon, Feb 12, 1990 13:40

	PTH µgl	F1	F2	F2 - F1	Sr clearance	Sr Extraction	Vr 1 (Co)	V.R. 2 (Tin)	Vr2 - Vr1	B1
	0	0.182	0.209	2.70e-2	0.148	0.710	795	640	-155	145
2	0	0.350	0.384	3.40e-2	0.199	0.500	345	300	-45	120
3	0	0.333	0.365	3.20e-2	0.175	0.480	405	370	-35	135
4	0	0.223	0.231	8.00e-3	0.156	0.675	540	350	-190	120
5	4	0.230	0.266	3.60e-2	0.177	0.660	480	280	-200	110
6	4	0.116	0.342	2.26e-1	0.183	0.535	1035	205	-830	120
7	4	0.226	0.399	1.73e-1	0.240	0.601	660	250	-410	150
8	4	0.356	0.320	-3.60e-2	0.181	0.565	420	390	-30	150
9	8	0.326	0.299	-2.70e-2	0.204	0.680	460	300	-160	150
10	8	0.406	0.315	-9.10e-2	0.284	0.900	350	220	-130	140
11	8	0.260	0.369	1.09e-1	0.167	0.450	500	220	-280	130
12	8	0.266	0.247	-1.90e-2	0.181	0.730	490	240	-250	130
13	12	0.296	0.387	9.10e-2	0.214	0.553	440	170	-270	130
14	12	0.313	0.122	-1.91e-1	0.126	1.030	415	580	165	130
15	12	0.276	0.115	-1.61e-1	0.076	0.661	380	520	140	105
16	12	0.466	0.099	-3.67e-1	0.065	0.660	300	860	560	140
17	12	0.293	0.137	-1.56e-1	0.117	0.860	530	580	50	155

	B2	B3/B4	B4 - B2	Muscle f1	Muscle F2	Muscle F2 - F1	Muscle V.R.1	Muscle V.R.2	Muscle V.R.2 -	F2 (expt)
1	145	145	0	0.065	0.134	6.84e-2	2225	1085	-1.14e+3	
2	115	115	0	0.064	0.084	2.00e-2	1875	1370	-5.05e+2	
3	135	135	0		0.324			420		
4	80	80	0	0.088	0.084	-4.00e-3	1364	950	-4.14e+2	
5	150	125	-25	0.050	0.185	1.35e-1	2200	405	1.85e+2	0.266
6	115	75	-40	0.161	0.113	-4.80e-2	745	620	-1.34e+2	0.342
7	125	70	-55	0.106	0.172	6.60e-2	1415	580	-8.35e+2	0.399
8	150	100	-50	0.142	0.110	-3.20e-2	1056	1140	8.40e+1	0.320
9	150	90	-60	0.097	0.119	2.20e-2	1550	750	-8.00e+2	0.299
10	130	70	-60	0.056	0.398	3.42e-1	2500	175	-2.32e+3	0.315
11	130	80	-50	0.142	0.524	3.81e-1	910	153	-7.57e+2	0.369
12	130	60	-70	0.159	0.366	2.07e-1	820	165	-6.55e+2	0.247
13	130	65	-65	0.093	0.433	3.40e-1	1400	150	-1.25e+3	0.387
14	130	70	-60	0.075	0.156	8.10e-2	1735	450	-1.28e+3	0.122
15	105	60	-45	0.317	0.170	-1.47e-1	330	360	3.00e+1	0.115
16	155	85	-70	0.120	0.140	2.03e-2	1180	610	-5.70e+2	0.099
17	160	80	-80	0.187	0.843	6.56e-1	830	95	-7.35e+2	0.137
18										

PGE2 Appendix

Tue, Feb 13, 1990 13:06

	PGE2	F1	F2	F2 - F1	Sr clearance	Sr Extraction	V.R. 1 (Co)	V.R. 2 (Tin)	VR 2 - VR 1	B1
1	0	0.429	0.421	-8.00e-3	0.322	0.765	315	321	6.00e+0	135
2	0	0.594	0.506	-8.80e-2	0.339	0.670	236	280	4.40e+1	140
3	0	0.358	0.429	7.10e-2	0.299	0.697	237	235	-2.00e+0	85
4	0	0.578	0.632	5.40e-2	0.317	0.510	208	190	-1.80e+1	120
5	20	0.256	0.232	-2.40e-2	0.348	1.500	410	345	-6.50e+1	105
6	20	0.347	0.274	-7.30e-2	0.315	1.150	375	290	-8.50e+1	130
7	20	0.319	0.215	-1.04e-1	0.234	1.090	425	440	1.50e+1	135
8	20	0.342	0.192	-1.50e-1	0.229	1.190	395	390	-5.00e+0	135
9	40	0.244	0.111	-1.33e-1	0.217	1.950	490	630	1.40e+2	120
10	40	0.411	0.213	-1.98e-1	0.283	1.320	315	375	6.00e+1	130
11	40	0.420	0.210	-2.10e-1	0.296	1.400	310	428	1.18e+2	130
12	40	0.150	0.133	-1.70e-2	0.220	1.600	735	450	-2.85e+2	110
13	60	0.234	0.127	-1.07e-1	0.235	1.800	555	670	1.15e+2	130
14	60	0.285	0.170	-1.15e-1	0.230	1.350	490	530	4.00e+1	140
15	60	0.540	0.147	-3.93e-1	0.250	1.700	300	480	1.80e+2	160
16	60	0.281	0.103	-1.78e-1	0.161	1.560	480	775	2.95e+2	135
17	80	0.374	0.199	-1.75e-1	0.250	1.200	320	350	3.00e+1	120
18	80	0.285	0.129	-1.56e-1	0.231	1.800	440	890	4.50e+2	125
19	80	0.340	0.187	-1.53e-1	0.254	1.400	380	535	1.55e+2	130
20	80	0.440	0.153	-2.87e-1	0.159	1.040	320	490	1.70e+2	140

	B2	B3	B4	B4 - B2	Muscle F1	Muscle F2	MF2 - MF1	Muscl V.R.1	Muscle V.R.2	M VR2 - VR1
1	135	135	135	0.00e+0	0.513	0.569	5.61e-2	263.000	240.000	-2.30e+1
2	135	140	140	5.00e+0	0.109	0.084	-2.50e-2	1285.000	1670.000	3.85e+2
3	85	100	100	1.50e+1	0.138	0.084	-5.40e-2	615.000	1190.000	5.75e+2
4	120	120	120	0.00e+0	0.088	0.084	-4.00e-3	1360.000	1430.000	7.00e+1
5	105	80	70	-3.50e+1	0.082	0.105	2.31e-2	1280.000	760.000	-5.20e+2
6	135	80	70	-6.50e+1	0.094	0.152	5.81e-2	1380.000	525.000	-8.55e+2
7	135	95	60	-7.50e+1	0.138	0.131	-7.00e-3	980.000	725.000	-2.55e+2
8	140	75	50	-9.00e+1	0.122	0.116	-6.00e-3	1105.000	645.000	-4.60e+2
9	120	70	70	-5.00e+1	0.084	0.272	1.88e-1	1430.000	260.000	-1.17e+3
10	130	80	75	-5.50e+1	0.228	0.139	-8.90e-2	570.000	575.000	5.00e+0
11	120	90	65	-5.50e+1	0.077	0.161	8.40e-2	1690.000	560.000	-1.13e+3
12	110	60	60	-5.00e+1	0.030	0.173	1.43e-1	3605.000	345.000	-3.26e+3
13	140	85	80	-6.00e+1	0.367	0.425	5.80e-2	355.000	200.000	-1.55e+2
14	160	90	80	-8.00e+1	0.216	0.118	-9.80e-2	650.000	765.000	1.15e+2
15	160	70	60	-1.00e+2	0.202	0.119	-8.30e-2	790.000	590.000	-2.00e+2
16	135	80	65	-7.00e+1	0.112	0.131	1.90e-2	1205.000	610.000	-5.95e+2
17	120	70	65	-5.50e+1	0.248	0.120	-1.28e-1	504.000	580.000	7.60e+1
18	125	115	75	-5.00e+1	0.269	0.113	-1.56e-1	465.000	1020.000	5.55e+2
19	125	100	70	-5.50e+1	0.124	0.163	3.85e-2	1045.000	610.000	-4.35e+2
20	130	75	70	-6.00e+1						

Calcitonin Appendix

	U./ animal	F1	F2	F2 -F1	Sr clearance	Sr extraction	V.R 1(Co)	Muscle F2	MF2 -MF1	Muscle V.R.1
1	0	0.217	0.259	4.20e-2	0.197	0.761	645	0.097	2.10e-2	1840.000
2	0	0.262	0.279	1.70e-2	0.222	0.796	460	0.091	2.50e-2	1820.000
3	0	0.359	0.227	-1.32e-1	0.178	0.784		0.089	2.40e-2	
4	0	0.305	0.259	-4.60e-2	0.178	0.687	360	0.072	-6.60e-2	80.000
5	0	0.318	0.397	7.90e-2	0.219	0.550	380	0.068	-6.00e-3	1620.000
6	10	0.291	0.259	-3.20e-2	0.178	0.690		0.019	-1.35e-1	
7	10	0.326	0.373	4.70e-2	0.252	0.675	415	0.143	1.20e-2	1030.000
8	10	0.268	0.176	-9.20e-2	0.167	0.950	450	0.023	-7.00e-3	4000.000
9	10	0.286	0.206	-8.00e-2	0.253	1.230	470	0.106	-6.00e-3	1205.000
10	10	0.357	0.181	-1.76e-1	0.181	1.000	490	0.110	-1.20e-2	1403.000
11	10	0.277	0.266	-1.10e-2	0.180	0.677	400	0.116	1.00e-2	1040.000
12	25	0.575	0.306	-2.69e-1	0.221	0.720	295	0.039	-2.30e-2	2740.000
13	25	0.554	0.455	-9.90e-2	0.265	0.580	290	0.068	-1.90e-2	1840.000
14	25	0.365	0.365	0.00e+0	0.193	0.530	355	0.087	-1.20e-2	
15	25	0.404	0.343	-6.10e-2	0.155	0.452		0.094	2.00e-2	1760.000
16	25	0.349	0.119	-2.30e-1	0.134	1.120	415	0.118	4.70e-2	2040.000
17	50	0.334	0.346	1.20e-2	0.289	0.835	390	0.097	-3.10e-2	1015.000
18	50	0.293	0.324	3.10e-2	0.158	0.490	480			
19	50	0.447	0.607	1.60e-1	0.330	0.540	335	0.146	5.30e-2	1610.000
20	50	0.194	0.324	1.30e-1	0.262	0.810	695	0.253	1.36e-1	1155.000
21	50	0.178	0.464	2.86e-1	0.274	0.600	840	0.097	-5.50e-2	990.000

Muscle V.R. 2	M VR2 - VR1	F2 (expt)	Sr clear (expt)	V.R.2 (Tin)	Vr2 - VR1	B1	B2	B3/B4	B4- B2	Muscle F1
1450	-3.90e+2			540	-1.05e+2	140	140	140	0	0.076
1370	-4.50e+2			450	-1.00e+1	120	125	120	-5	0.066
										0.065
1530	1.45e+3			425	6.50e+1	110	110	110	0	0.138
1620	0.00e+0			280	-1.00e+2	120	110	110	0	0.074
		0.259	0.178							0.154
910	-1.20e+2	0.373	0.252	350	-6.50e+1	135	130	130	0	0.131
5220	1.22e+3	0.176	0.167	680	2.30e+2	120	120	120	0	0.030
1230	2.50e+1	0.206	0.253	630	1.60e+2	135	130	130	0	0.112
1455	5.20e+1	0.181	0.181	885	3.95e+2	175	160	160	0	0.122
950	-9.00e+1	0.266	0.180	415	1.50e+1	110	110	110	0	0.106
4360	1.62e+3	0.306	0.221	555	2.60e+2	170	170	170	0	0.062
2350	5.10e+2	0.455	0.265	350	6.00e+1	160	160	160	0	0.087
		0.365	0.193	360	5.00e+0	130	130	130	0	0.099
1380	-3.80e+2	0.343	0.155							0.074
850	-1.19e+3	0.119	0.134	840	4.25e+2	145	100	100	0	0.071
1340	3.25e+2	0.346	0.289	375	-1.50e+1	130	130	130	0	0.128
		0.324	0.158	415	-6.50e+1	140	135	140	5	
925	-6.85e+2	0.607	0.330	222	-1.13e+2	150	135	135	0	0.093
535	-6.20e+2	0.324	0.262	415	-2.80e+2	135	135	135	0	0.117
1390	4.00e+2	0.464	0.274	290	-5.50e+2	150	135	135	0	0.152

	Flow 1	Flow 2	Sr Clearance
1	0.358	0.428	0.299
2	0.321	0.332	0.178
3	0.182	0.209	0.148
4	0.500	0.471	0.336
5	0.297	0.291	0.197
6	0.327	0.299	0.216
7	0.136	0.167	0.122
8	0.177	0.123	0.116
9	0.182	0.209	0.148
10	0.350	0.384	0.199
11	0.333	0.365	0.175
12	0.223	0.231	0.156
13	0.429	0.421	0.322
14	0.594	0.506	0.339
15	0.358	0.429	0.299
16	0.578	0.632	0.317
17	0.217	0.259	0.197
18	0.262	0.279	0.222
19	0.359	0.227	0.178
20	0.305	0.259	0.178
21	0.318	0.397	0.219

APPENDIX 2

Dose-Dependent Reduction of Bone Blood Flow and ^{85}Sr Clearance Caused by Prostaglandin E_2

E. Cochrane, R. H. Fleming, and I. D. McCarthy

Introduction

Osteolytic effects of series E prostaglandins are well established in vitro and in vivo (Klein and Raisz 1970; Vanderwiel and Talmage 1979), and it has also been reported that the injection of massive doses of native prostaglandin E_2 (PGE_2) into rats can enhance bone mineralisation (Uemo et al. 1984). Recent reports have claimed that 16-16 dimethyl PGE_2 produced inhibition of ^{45}Ca uptake in chicks. It was suggested that this represented a novel response, but the possibility of an indirect vascular mechanism was also discussed. In this paper, we have investigated the relationship between the inhibition of mineral uptake by PGE_2 and changes in bone blood flow. This has then been compared with the response due to the vasoactive effect of noradrenaline.

Materials and Methods

The study involved the use of radioactive microspheres and labelled isotopes to establish bone blood flow and strontium clearance measurements. After dissection, a cannula was inserted within the heart via the carotid artery and a second cannula inserted into the tail artery. Microspheres were inserted into the carotid cannula and reference blood samples were withdrawn from the tail cannula. A control flow rate was established using ^{57}Co microspheres. Blood was withdrawn from the tail artery at a rate of 0.197 ml/min, and 30 s later the microspheres were injected via the carotid cannula. Withdrawal of blood from the tail artery ceased after a total of 2 min. A stock solution of PGE_2 was made by dissolving it in ethanol, and this was then made into test solutions of 0, 20, 40, 60 and 80 μg of PGE_2 using Krebs-Ringer buffer solution containing 1% albumin. Each dose group contained six Sprague-Dawley rats weighing approximately 300–350 grams. The test solution was injected via the carotid cannula. After 30 s blood was withdrawn from the tail artery. ^{113}Sn microspheres, 15 μm in diameter, and ^{85}Sr were injected via the carotid artery 30 s later. Withdrawal of blood from the tail artery ceased after a total of 4.5 min.

This method was also followed using noradrenaline as the test solution. However, due to the properties of noradrenaline and its rate of metabolism in

the body the test solution was infused over the experimental period at a rate of 1.3 μg kg per min and not injected as a bolus. Animals were killed. both tibiae and femora were removed quickly to minimise post mortem migration. and bone and blood samples then measured for radioactivity.

Bone blood flow and ^{85}Sr clearance were calculated from the following equations:

$$\text{Bone blood flow} = \frac{\text{microsphere activity in bone} \times \text{pump rate}}{\text{microsphere activity in blood}}$$

$$^{85}\text{Sr clearance} = \frac{^{85}\text{Sr activity in bone} \times \text{pump rate}}{^{85}\text{Sr activity in blood}}$$

Results

The results presented in Figs. 1 and 2 show that PGE_2 produced a dose-dependent reduction of both blood flow and ^{85}Sr clearance. In each dose group the significance of the effect of PGE_2 on bone blood flow was assessed by comparing the paired values for the first and second flow measurements. This highlighted a general trend. a decrease in bone blood flow with increasing PGE_2 concentration. PGE_2 decreased flow to bone from a control value of 0.3 ml/min per g to 0.095 ml/min per g at PGE_2 concentration of 80 μg per rat. Using the student's paired t test the flow changes which are significant are those at

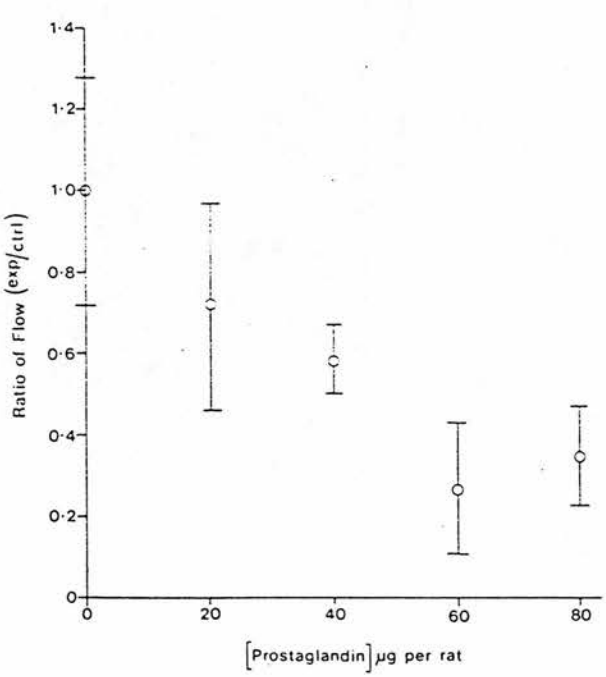

Fig. 1. Change in flow rate with injected prostaglandin E_2 for rat bone (mean ±SEM)

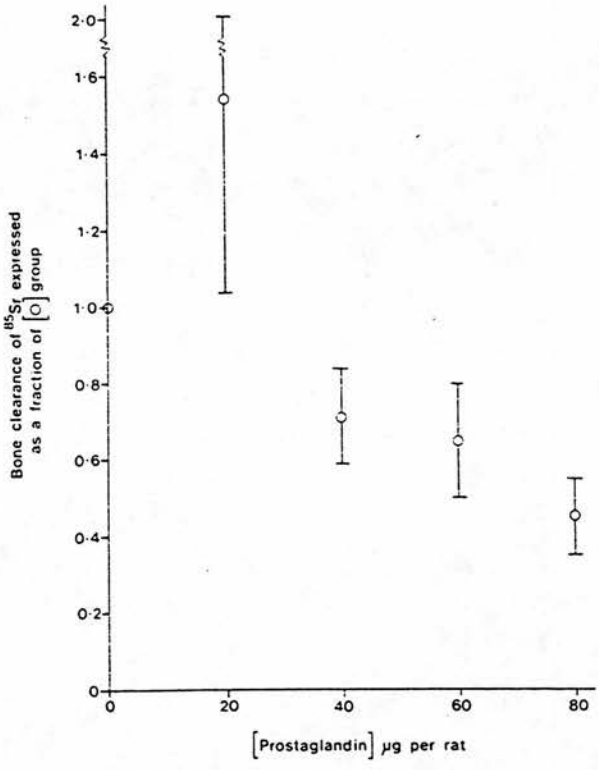

Fig. 2. Change in strontium clearance with injected prostaglandin E_2 for rat bone (mean ± SEM)

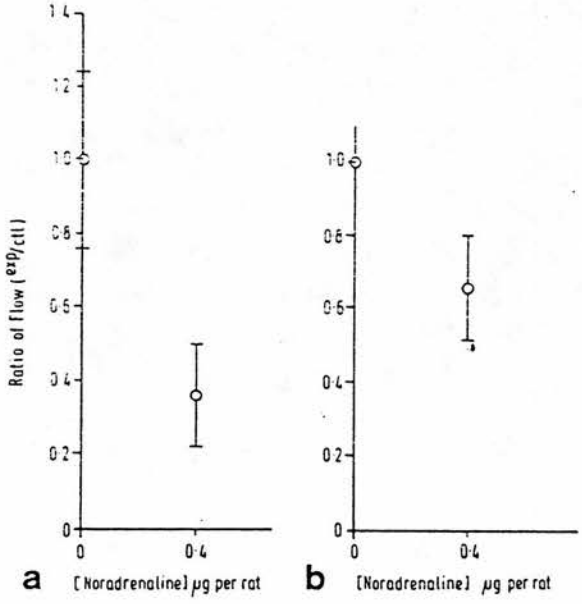

Fig. 3. a Change in flow rate with noradrenaline infusion for rat bone (mean ± SEM). b Effect of noradrenaline on strontium clearance

40 and 80 μg PGE_2 dose groups ($P<0.02$). Comparison of the control mean ^{85}Sr value with each of the PGE_2 dose group shows the same trend, a dose-dependent reduction of strontium clearance (Fig. 2). Statistical analysis shows that strontium clearance at 80 μg dose group is significantly different from control ($P<0.01$). The effect of noradrenaline infusion on flow and clearance are shown in Fig. 3. Noradrenaline at a concentration of 1.3 μg/kg per min decreased both bone blood flow and ^{85}Sr clearance, its effect on flow and clearance being similar to higher doses of PGE_2.

Discussion

The results show a pattern of decreasing bone blood flow and ^{85}Sr clearance with increasing PGE_2 concentration, with the one concentration of noradrenaline having a similar effect. Tothill and Hooper (1984) and Schoutens et al. (1979) have previously shown clearance is not linearly related to bone blood flow and that bone clearances become poor indicators of increases of bone blood flow. The data presented here are in agreement with these results, suggesting there is no apparent difference between the effects of PGE_2 and noradrenaline. If it is assumed that the noradrenaline acts only by increasing peripheral vascular resistance and reducing blood flow, then it suggests the reduction of clearance by PGE_2 is also an indirect vascular effect. There is no "novel" inhibition of calcium uptake. The mechanisms of reducing blood flow are not necessarily the same as PGE_2 lowers blood pressure, noradrenaline increases blood pressure.

In summary, PGE_2 and noradrenaline infusion cause changes in both bone and ^{85}Sr clearance. The results suggest that changes in ^{85}Sr clearance are dependent on the effect of the drugs on bone blood flow.

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APPENDIX 3

Effect of Calcium Regulating Hormones and Vasoactive Substances on Bone Blood Flow

E. Cochrane and Dr. I.D. McCarthy

Dept. of Orthopaedic Surgery, University of Edinburgh

It is now believed that there is a significant association between skeletal blood flow and bone turnover. We have been investigating the action of vasoactive substances, noradrenaline and ATP and calcium regulating agents, PGE2, PTH and calcitonin on bone blood flow within a five minute period. Radioactive microspheres were used to estimate blood flow and strontium-85 for strontium clearance in rats weighing approximately 350 grammes.

Calcitonin produced no effect on blood flow or pressure but significantly increased strontium clearance ($p<0.03$). Administration of PGE2 produced a dose dependent reduction of blood flow, pressure and strontium clearance ($p<0.006$). Similarly PTH significantly reduced blood pressure and blood flow in a dose dependent fashion ($p<0.003$). Infusion of noradrenaline resulted in a significant decrease in blood flow ($p<0.01$) and an increase in blood pressure ($p<0.003$). While ATP caused a significant decrease in blood pressure.

With all groups, there was a significant relationship between blood flow and strontium clearance, but the slope of regression was significantly different for PGE2 ($p<0.01$). This shows that PGE2 was having a direct effect on bone independent of its effect on blood flow. It also shows that clearance values are not appropriate for the measurement of bone blood flow.

APPENDIX 4

The Effects of vaso-active substances and calcium regulating hormones on bone blood flow and strontium clearance

I.D. McCarthy, E. Cochrane and S.P.F. Hughes
Department of Orthopaedic Surgery, University of Edinburgh,
Edinburgh EH8 9AG, U.K.

INTRODUCTION: It has been recently reported that parathyroid hormone (PTH) and prostaglandin E2 (PGE2) produce a very rapid inhibition of calcium uptake in chicks (1). In order to investigate whether this is the result of a vascular effect, or whether this is a direct effect on bone, the response of bone blood flow and strontium clearance to PTH, PGE2 and calcitonin in rats has been measured. These were then compared with the responses due to noradrenaline and ATP.

MATERIALS AND METHODS: Sprague Dawley rats, weighing approximately 300 to 350g, were used for these experiments. After dissection, a cannula was inserted within the heart via the carotid cannula, and a second cannula inserted into the tail artery. An initial control blood flow measurement was performed. Blood was withdrawn from the tail artery at a rate of 0.197ml/min, and thirty seconds after start of withdrawal microspheres labelled with cobalt-57 were injected via the carotid cannula. Withdrawal of blood was stopped after 2 minutes. At this point either PGE2 (20, 40, 60, 80μg), PTH (4, 8, 12μg), calcitonin (10, 15, 25U.), noradrenaline (1.0, 1.5, 2.0 μg), ATP (10.25, 0.75, 1.25mg) or an equal volume of Krebs Ringer buffer was administered. One minute later, blood was withdrawn from the tail artery, and thirty seconds later, microspheres labelled with tin-113, together with strontium-85, were injected via the carotid cannula . Blood was withdrawn from the tail artery for a period of 4.5. minutes. The animals were then sacrificed and both tibiae and femora were removed very quickly, so that post mortem migration of strontium into the bones were minimised. The bones and both blood samples for radioactivity. The total number of microspheres in the four bones was normally about 1000. Blood flow and strontium clearance were calculated from the radioactivity measurements using the standard equations.

RESULTS: ATP did not produce any direct effect on bone. Noradrenaline produced a dose dependent decrease of bone blood flow ($p=0.026$), and a related increase in vascular resistance ($p\leq 0.01$). However, there was no change in the strontium clearance.

PGE2 also produced a dose related decrease of bone blood flow ($p\leq 0.001$) and an increase of vascular resistance. However, a significant decrease ($p\leq 0.005$) in strontium clearance was also observed (see Figure 1). Although administration of PTH resulted in a decrease in blood flow, this was not associated with any significant change in vascular resistance or strontium clearance. Calcitonin, while not having a significant effect, did slightly increase strontium clearance ($p\leq 0.03$).

The relationship between strontium clearance and blood flow was generally linear in the range of values measured. However, there is a significant difference ($p\leq 0.01$) between the slopes of the regression lines for control rats (0.47) and those rats given PGE2 (0.78). In all other groups, the relationship between blood flow and clearance was indistinguishable from the control group.

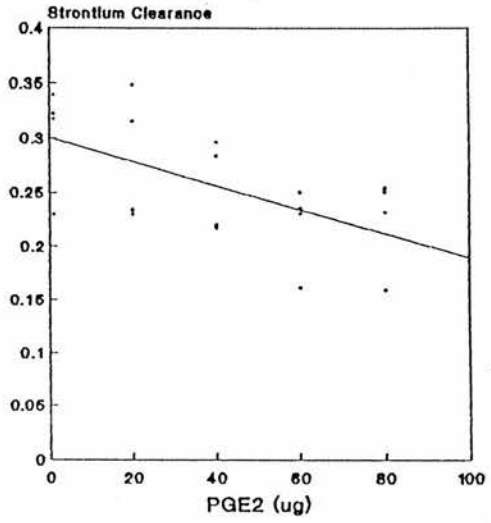

Figure 1. The relationship between strontium clearance and PGE2 concentration.

DISCUSSION: These data show that PGE2 has an immediate direct effect on bone, in addition to its effect on blood flow. Previous work has shown that metabolic activity could alter the immediate distribution of strontium-85 in bone, either by controlling access to a compartment or by increased capacity for binding to bone surfaces (2), and the present data support that idea.

Clearance measurements have been used for bone blood flow measurement. However, the extraction of bone-seeking tracers is flow dependent. The present work shows that there are additional factors that control extraction, and great care is needed in extrapolating from clearance to bone blood flow.

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